PHD

Development of a human model system to monitor complement-mediated lysis in myasthenia gravis

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DEVELOPMENT OF A HUMAN MODEL SYSTEM TO MONITOR COMPLEMENT-MEDIATED LYSIS IN MYASTHENIA GRAVIS.

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For the degree of Ph.D. of the

University of Bath

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For my late father, with love and thanks.

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Finally, I would like to thank the numerous members of the "Ward family", especially my mother, for their continuing support throughout my extended education.

SUMMARY.

The involvement of antibody-mediated, complement-dependent cell lysis in myasthenia gravis was investigated in vitro by using two sources of human-derived cell.

Lysis of human muscle cells in culture, was assayed by following the release of tritium-labelled carnitine selectively taken up by these cells. In the presence of exogenous complement, heat-inactivated serum from 5 myasthenic patients, showed greater myotoxicity than did normal human serum, indicating the involvement of a complement-mediated effect in MG. More rigorous investigation of this system was hindered by problems arising from the sporadic supply of uncontaminated human muscle and an alternative source of human cells was sought.

A pharmacological profile of TE671 cells, using [^{125}I] α-BGT and other cholinergic ligands, established the cell line to be a carrier of a "muscle-type" AChR. The cells were accordingly used in cytotoxicity studies of complement-mediated lysis.

An assay involving release of intracellular lactate dehydrogenase (LDH) was initially examined, but despite many refinements, consistently higher lysis by myasthenic serum, compared with controls, could not be demonstrated. An alternative assay, depending on the release of [^{51}Cr] from the cells gave similar results

leading to the eventual conclusion that TE671 cells, while readily lysed by hypotonic shock or detergent were intrinsically resistant to rupture by anti-AChR antibodies and complement.

PUBLICATIONS.

Communications.

Ward, J.M., Harrison, R., Lunt, G.G. and Childs, L.A. (1986) Pathogenicity of myasthenic serum on cultured human myotubes. Biochem. Soc. Trans., 14, 1233-1234.

Ward, J.M., Harrison, R., Lunt, G.G. and Childs, L.A. (1986) Pathogenic effects of myasthenic serum on cultured human myotubes. Biological Chemistry, Hoppe-Seyler, 367, 1123-1124.

Ward, J.M., Harrison, R. and Lunt, G.G. (1987) Inhibition of [^{125}I] α-bungarotoxin binding to TE671 cells using sera from patients with myasthenia gravis. J. Neurochem., 48, Suppl. S86D.

Ward, J.M., Harrison, R. and Lunt, G.G. (1989) Pharmacological assessment of [^{125}I] α-bungarotoxin binding to TE671 cells. ICSU Short Report, 9, Advances in gene technology: Molecular neurobiology and neuropharmacology.

Ward, J.M., Harrison, R. and Lunt, G.G. (1989) TE671 cells: potential use as a source of acetylcholine receptor for studies of myasthenia gravis. 2nd Eur. Conference on MG, Abstr. p54.

ABBREVIATIONS.

Ab	Antibody
mAb	Monoclonal antibody
ACh	Acetylcholine
AChE	Acetylcholine esterase
AChR	Acetylcholine receptor
α-BGT	α-Bungarotoxin
BSA	Bovine serum albumin
BZQ	Benzoquinonium chloride
C	Complement component
Carb	Carbachol
cys	Cysteine
DHS	Donor horse serum
cDNA	Complementary deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
EDTA	Ethylene diamine tetra-acetic acid
epp	End plate potential
FCS	Fetal calf serum
GPC	Guinea pig complement
Hagg	Heat aggregated immunoglobulin G

HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethylpiperazine -N'-2-ethane sulphonic acid
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LDH	Lactate dehydrogenase [EC 1.1.1.27.]
MAC	Membrane attack complex
mepp	Miniature end plate potential
MIR	Main immunogenic region
MLA	Methyllycaconitine
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PMSF	Phenylmethylsulphonyl fluoride
RIA	Radioimmunoassay
mRNA	Messenger ribonucleic acid
d-TC	d-Tubocurarine chloride
Triton X-100	Isooctyl phenoxypolyethoxyethanol

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INTRODUCTION.

Myasthenia gravis (MG) was first described in 1672 by Dr. Thomas Willis. Since then, and especially in the last 25 years, a great deal of information concerning the disease has been recorded (for reviews, see Vincent, 1980; Harrison and Behan, 1986). Clinically, MG is characterised by weakness and fatiguability of skeletal muscle, with a tendency to be made worse by activity.

It is now accepted that MG is an autoimmune disease and the nicotinic acetylcholine receptor (AChR) at the neuromuscular junction has been demonstrated to be the major autoantigen. Elevated levels of anti-(AChR) antibodies are found in the serum of about 90% of patients with the disease, which results in a loss of functional AChR and impairment of neuromuscular transmission. Understanding of the pathogenesis of MG is coupled to increasing knowledge of synaptic organisation and to structure and function of the AChR. In the generation of this knowledge, tissue culture systems and cell lines have proved valuable tools.

1.1 Neuromuscular transmission.

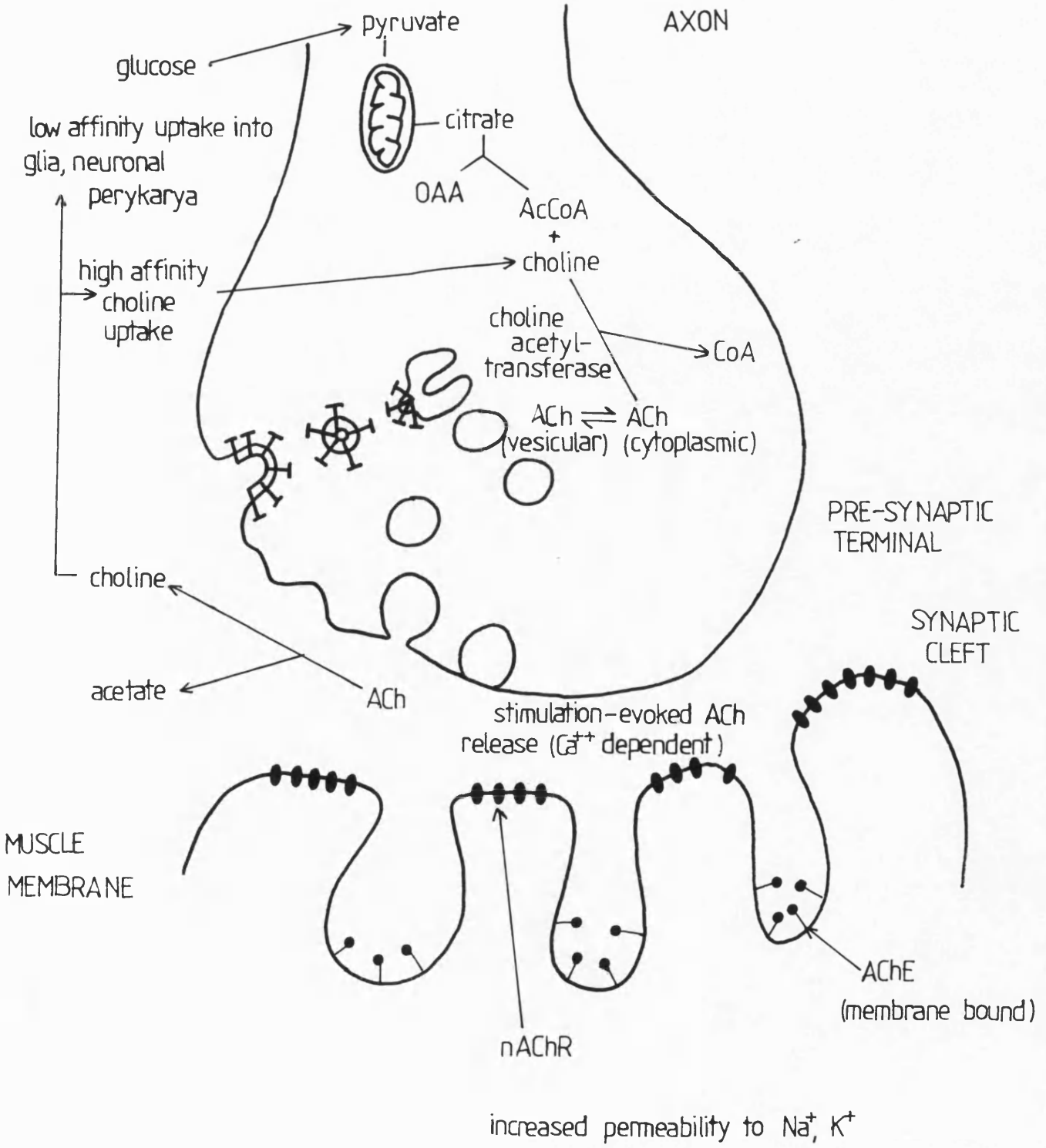
In the majority of mammalian muscles, each muscle fibre has a single region of contact with the axon of

its controlling motor neurone. This region constitutes the neuromuscular junction (Fig. 1). Its function is to transfer the propagated nerve impulse from the motor nerve ending to the muscle fibre, resulting ultimately in muscular contraction. Although the nerve terminal and the post-synaptic membrane lie close together they are separated by a gap that must be bridged by acetylcholine in order to generate an action potential in the muscle fibre.

The sequence of events which results in muscular contraction may be summarised as follows:-

An action potential is propagated along the membrane of the motor neurone and invades the nerve terminal. Depolarisation of the terminal causes the release of quanta of acetylcholine. Each quantum contains approximately 10^6 molecules and is thought to be the contents of one synaptic vesicle. The vesicles are concentrated in the motor nerve terminal close to the post-synaptic membrane. The liberated acetylcholine rapidly diffuses across the synaptic cleft and binds to receptors on the crests of the folded post-synaptic muscle membrane. The binding of acetylcholine to the AChR in the post-synaptic membrane promotes a conformational change, associated with a brief (1 millisecond) opening of an ion channel. This allows the passage of sodium and potassium ions down their electrochemical gradients. There is a net influx of sodium ions, resulting in a reduction of membrane potential or depolarisation of the muscle membrane,

FIG. 1 SCHEMATIC REPRESENTATION OF THE NEUROMUSCULAR JUNCTION



producing an "end-plate potential" (epp). If the summation of epps causes depolarisation to the threshold value, the muscle membrane generates an action potential which ultimately activates the contractile processes of the muscle fibre.

Normally, the amount of acetylcholine released and the number of AChRs activated is much larger than the minimum required to initiate muscular contraction. This provides a large safety factor, ensuring effective neuromuscular transmission. In the absence of a nerve impulse, spontaneous release of a small amount of acetylcholine from the nerve ending occurs, generating small depolarisations - miniature end-plate potentials (mepps). These potentials are not large enough by themselves to produce muscle action potentials, and are thought to be the result of the random bursting of single vesicles.

The action of acetylcholine is terminated by its dissociation from the AChR, subsequent reassociation is minimised by acetylcholinesterase activity.

1.2 Molecular characterisation of AChR.

Since the early 1900's, two classes of AChR, muscarinic and nicotinic, have been recognised; the classes are based on the differential effects of the alkaloids muscarine and nicotine (Dale, 1914; Dixon, 1907). While both muscarinic and nicotinic receptors

have a common neurotransmitter in acetylcholine, that is where the similarity ends.

The state of knowledge of the nicotinic AChR is more advanced than for any other type of receptor. Much of the progress in generating knowledge about this "archetypal" receptor protein can be attributed to four factors:-

i) The discovery of an abundant source of AChR in the elasmobranch and teleost fishes (Torpedo and Electrophorus sp.).

ii) The use of snake α-neurotoxins, principally α-bungarotoxin (α-BGT), as specific, high affinity probes for the AChR at the neuromuscular junction.

iii) The ability of detergents to solubilise the protein from its membrane environment without loss of biological activity.

iv) The more recent application of the sophisticated and powerful techniques of molecular biology, including the translation system of the Xenopus oocyte.

1.2.1. Source of AChR:-

1.2.1.1. Torpedo as a model.

The majority of information concerning the AChR has come from studies on Torpedo electric organ, this work has proceeded to include mammalian AChR. Details

pertaining to the mammalian AChR will be included where appropriate.

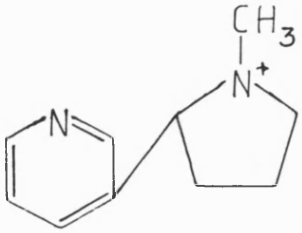
There are similarities between the AChR in the neuromuscular junction and those in the electric organs of Torpedo and Electrophorus. In Torpedo the embryonic fish contains muscle-like precursor cells that develop into the orientated stack of polar cells comprising the electric organs. These contain cells, electrocytes, that are the counterparts of muscle cells, but lack the contractile elements. On only one side of the electrocyte is the plasmalemma endowed with AChRs but the density is high (in Torpedo 25% of the total membrane protein). In contrast, in normal adult innervated muscle the AChR comprises less than 1% of the total membrane protein. AChRs from Torpedo are related by structure, function and evolution to those of the neuromuscular junction in vertebrates. The electric organ is, therefore, a rich and homogeneous source which has been used extensively for biochemical characterisations (for reviews, see Karlin, 1980; Conti-Tronconi and Raftery, 1982; Popot and Changeux, 1984; McCarthy et al., 1986; Hucho, 1986). However, after denervation of vertebrate muscle, the number of AChRs increases up to 50-fold, providing a useful and enriched source of receptors which has facilitated their purification and chacterisation (for review, see Dolly and Barnard, 1984).

1.2.1.2. Snake α-neurotoxins.

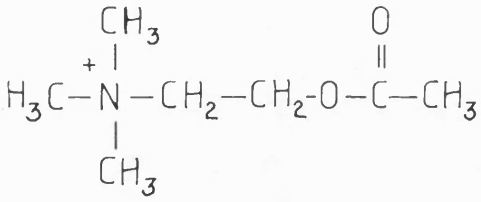
The neurotoxins were first purified by Chang and Lee (1962) who demonstrated that they produce an anti-depolarising block of the AChR at the neuromuscular juction, in a manner similar to that of d-tubocurarine, a cholinergic antagonist (Lee, 1972). Although their structure is very different from those of acetylcholine analogues (Fig. 2), the α-neurotoxins bind non-covalently but with high affinity (Kd 10^{-11}M) and specificity, at or near the acetylcholine binding site (Section 1.2.4.4.).

Their usefulness as probes has relied on the finding that they can be labelled with radioactive, fluorescent or enzyme markers. α-BGT from Bungarus multicinctus is normally used because of the near irreversibility of its binding to AChR (Lee,1972) and the ease with which it can be labelled without loss of biological activity. α-BGT can be radioiodinated with [^{125}I] to high levels of specific radioactivity, and subsequently separated from the unlabelled toxin (Section 2.1.). Fluorescent derivatives of α-BGT, prepared by reaction with tetramethyl-rhodamine isothiocyanate, are generally used for studies on muscle, but fluorescein conjugates have also been used successfully. Finally, physiologically active conjugates of α-BGT and peroxidase can be made or, alternatively anti-(α-BGT) antibodies can be used in conjunction with a labelled second antibody (Barnard, 1979). The latter

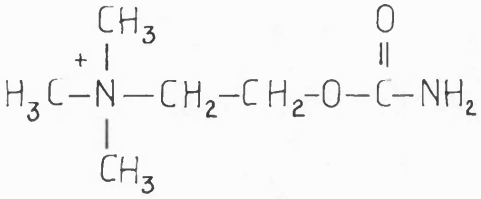
FIG. 2 Cholinergic effectors



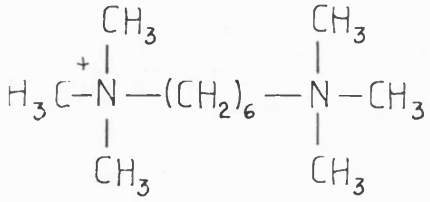
Nicotine



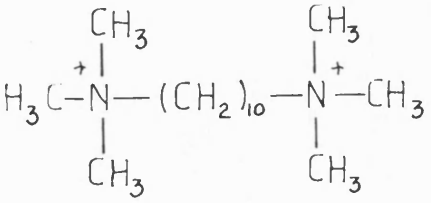
Acetylcholine



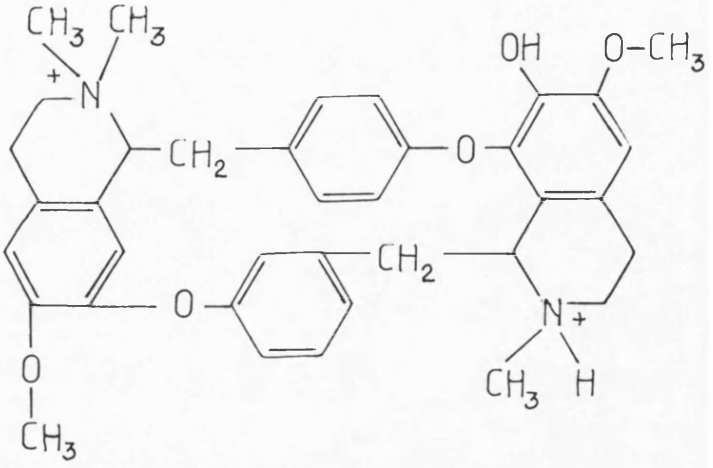
Carbamylcholine



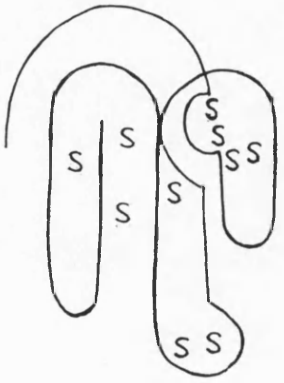
Hexamethonium



Decamethonium



d-Tubocurarine



α-Bungarotoxin

techniques allow the AChR to be localised by using both light and electron microscopy (Barnard, 1979).

1.2.2. Protein chemistry.

1.2.2.1. Isolation and purification of AChR.

The AChR is an integral membrane protein that can be extracted with non-ionic detergent (eg: Triton X-100). In solution as detergent micelles it is still capable of binding α-toxins and cholinergic ligands. Further purification is now usually effected by using the less avid α-toxin from Naja naja siamensis and elution with cholinergic ligands. Purified AChR is freed from eluting agents by standard techniques such as dialysis or ion-exchange chromatography.

Purification of mammalian AChR proved more difficult than for the electroplaque AChR, mainly because of the much lower content of AChR in the muscle membrane (2500-fold less than its piscine counterpart, Dolly and Barnard, 1984) and the high level of proteolysis that occurs during purification (for reviews, see Dolly, 1979; Dolly and Barnard, 1984).

1.2.3. Properties of the isolated AChR.

1.2.3.1. Molecular weight.

The subunit composition of the purified Torpedo AChR has been determined by denaturation with sodium dodecyl sulphate (SDS) followed by polyacrylamide gel electrophoreris (PAGE). It is now generally agreed that the electric organ AChRs are comprised of four subunits, α, β, τ and δ with apparent molecular weights of approximately 40000, 49500, 57000 and 64000 Daltons in the stoichiometric ratio 2:1:1:1.

1.2.3.2. Reconstitution of AChR in model membranes.

Re-assembly of the detergent-solubilised, purified receptor into a membrane environment constitutes proof that the purified protein contains all the elements of a functional AChR. The AChR has now been reconstituted into lipid bilayer vesicles and lipid films with complete recovery of ligand binding and ion-permeability properties (Anholt, 1981; McNamee and Ochoa, 1982).

1.2.3.3. Molecular shape.

Most studies concerning the shape of the AChR molecule were carried out using Torpedo AChR. Since the AChR molecule, formed by the four subunits mentioned

above, contains both the binding sites for the cholinergic ligands and the cation gating system (for review, see Conti-Tronconi and Raftery, 1982), it should span the post synaptic membrane. A direct demonstration that the AChR molecule protrudes from both sides of the post synaptic membrane was obtained after treatment with proteolytic enzymes and subsequent utilisation of anti-(AChR) antibodies binding to post synaptic membrane fragments of Torpedo (Tarrab-Hazdai et al., 1978). Both the outside and inside surfaces were labelled by antibodies, thus demonstrating that the AChR is a transmembrane protein complex.

1.2.3.4. Electron microscopy.

The five membrane-spanning subunits of the AChR have been resolved in electron microscope images (Brisson and Unwin, 1985) and are shown to lie at pentagonally symmetrical positions in a ring-like structure (8nm diameter), around a densely stained central pit (2.5nm diameter), the presumed ion-channel (Hucho, 1986). The channel consists of a wide synaptic portion and a narrow portion extending through the membrane into the interior of the cell (Fig. 3).

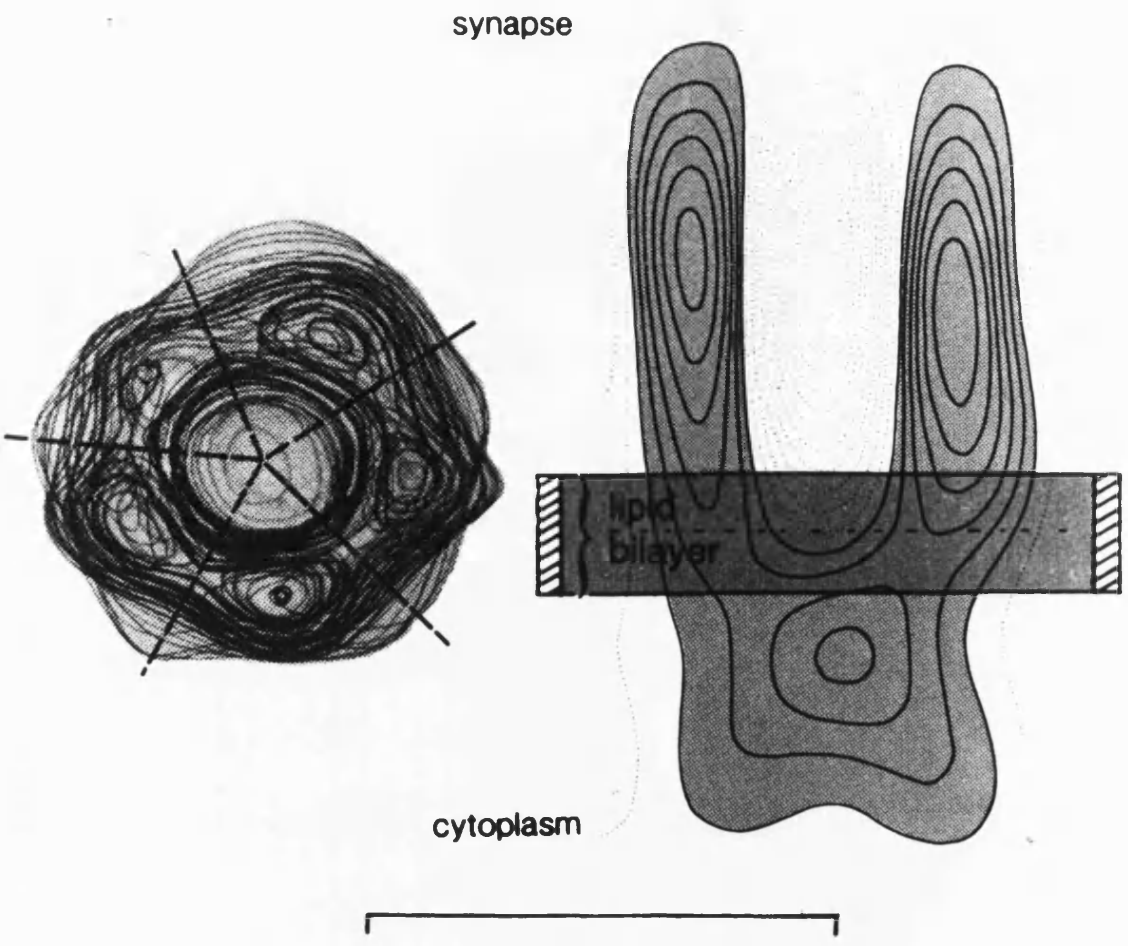
Fig. 3.

a) A 3D electron image map of receptor molecules in crystals grown from native Torpedo membrane vesicles viewed as they would appear from the synaptic cleft. The receptor is composed of a pentagonally symmetrical array of 5 subunits of similar size, lying perpendicular to the membrane plane. Thus, all five subunits are equally spaced around a pore, thought to be the ion channel and are orientated in a similar fashion in the cell membrane.

b) Side view (through the channel axis) with the central plane of the bilayer shown by a broken line. In this section, it can be seen that the receptor is a cylindrical molecule of almost constant diameter, the ion channel opening is wide at the synaptic end of the molecule and has an unresolved part extending through the membrane into the cytoplasm.

Taken from Brisson and Unwin (1985).

Fig.3



1.2.4. Molecular biology.

1.2.4.1. Cloning and sequencing AChR subunits: homologous polypeptides.

A prerequisite for understanding the molecular basis of the normal functions of the AChR was the elucidation of the primary structures of the constituent polypeptides of the receptor. One of the first descriptions of the amino acid compositions of the AChR came after Raftery et al., (1980) chemically sequenced the N-terminal 54-56 amino acids of the amino termini of the four polypeptides of T. californica and noticed considerable homology between subunits. Subsequently, Numa and his many co-workers (Noda et al., 1982) using the powerful recombinant DNA techniques, identified the mRNA for the α-subunit and cloned and sequenced the cDNA.

The same techniques allowed determination of the sequences of the β and δ subunit precursors and subsequently of the polypeptide chains for all four Torpedo subunits (Noda et al., 1983a, b; Claudio et al., 1983).

The application of recombinant DNA technology has also been used for further structural and functional analysis of mammalian AChR. Sequences of polypeptide chains for calf and human AChR subunits have been determined (Noda et al., 1983c ; Tanabe et al., 1984; Takai et al., 1984; Kubo et al., 1985; Shibihara et

al., 1985). Comparison of these subunits revealed extensive homologies throughout their lengths and also between species; there is about 80% homology between α-subunits of Torpedo and human muscle and about 55% homology of the other subunits between these species (Noda et al., 1983c; Takai et al., 1984; Tanabe et al., 1984: Kubo et al., 1985).

These homologies confirmed the earlier suggestion (Raftery et al., 1980) that the genes for the four polypeptide chains may be derived from a common ancestor. It has further been suggested that α/β diverged from τ/δ, and that the α-subunit subsequently diverged again later from β, τ and δ (Anderson, 1987).

In addition to the four subunits described above, a novel subunit, termed the ϵ subunit, has been discovered by cloning and sequencing calf muscle cDNA (Takai et al., 1985). This subunit shows greatest homology with the τ subunit and functional expression studies (see section 1.2.4.2. below) suggest that it replaces the τ subunit in adult muscle AChR.

1.2.4.2. Functional expression.

Expression of the subunit mRNA's into the Xenopus oocyte, showed that all four subunits are necessary for assembly and membrane insertion of a functional receptor (Mishina et al., 1985). Takai et al. (1985) showed that

the calf ϵ subunit can substitute for calf τ or Torpedo τ subunits to create a functional AChR. The functional system can be used to investigate ligand binding, electrophysiological properties and the effects of substitutions or deletions of amino acids. Takai et al. (1985) used this to advantage by demonstrating that substitution of ϵ for τ subunits resulted in altered ionic channel properties.

Mishina et al. (1986) suggested that different forms of the AChR occur in mammalian skeletal muscle during development. The AChR with the ϵ subunit is present predominantly at the neuromuscular junction in adult muscle, whereas in fetal muscle and also in denervated muscle, AChRs with τ subunits are found in the extrajunctional regions. These types of AChR differ in their channel properties and also in the number of acidic amino acid residues. Mishina et al. (1986) proposed that the developmental changes in the AChR channel properties are as a result of nerve-induced changes in AChR gene expression.

1.2.4.3. Models of AChR structure.

Comparison of the cDNA sequences for all four (α, β, τ and δ) gene products of Torpedo have revealed the following features. Each sequence contains four very hydrophobic sequences which form α-helices long enough

to form transmembrane domains (designated M1-M4) (Noda et al., 1983abc; Devillers-Thiery et al., 1983; Tanabe et al., 1984; Takai et al., 1984; Kubo et al., 1985; Shibahara et al., 1985), similar to those previously described for bacteriorhodopsin (Ovchinnikov et al., 1979). An additional sequence with alternating polar and hydrophobic amino acids has been recognised in all subunits. It was suggested that this might form an amphipathic transmembrane helix with the charged face contributing the ion-channel lining (Finer-Moore and Stroud, 1984). This suggestion has now been discredited (see below).

The use of monoclonal antibodies raised against whole subunits or parts of the AChR and the construction of synthetic peptides based on previously described sequences (see Lindstrom, 1986), have aided in the location of specific polypeptide sequences with respect to the membrane.

The precise transmembrane orientation of the AChR subunit polypeptide chains is uncertain, although several models have been proposed (Guy, 1986) (Fig. 4). It is evident that each subunit has a large amino terminal region on the synaptic face. This contains the sites for glycosylation (Anderson and Blobel, 1981) and, in the α subunits, contains the high affinity agonist and antagonist binding sites (Kao et al., 1984. See also Section 1.2.4.4.). Sites for phosphorylation have been demonstrated to be on the cytoplasmic side of the membrane (Huganir et al., 1984). Given that the N-

Fig. 4. Primary sequence and possible transmembrane configurations of nicotinic receptor subunits.

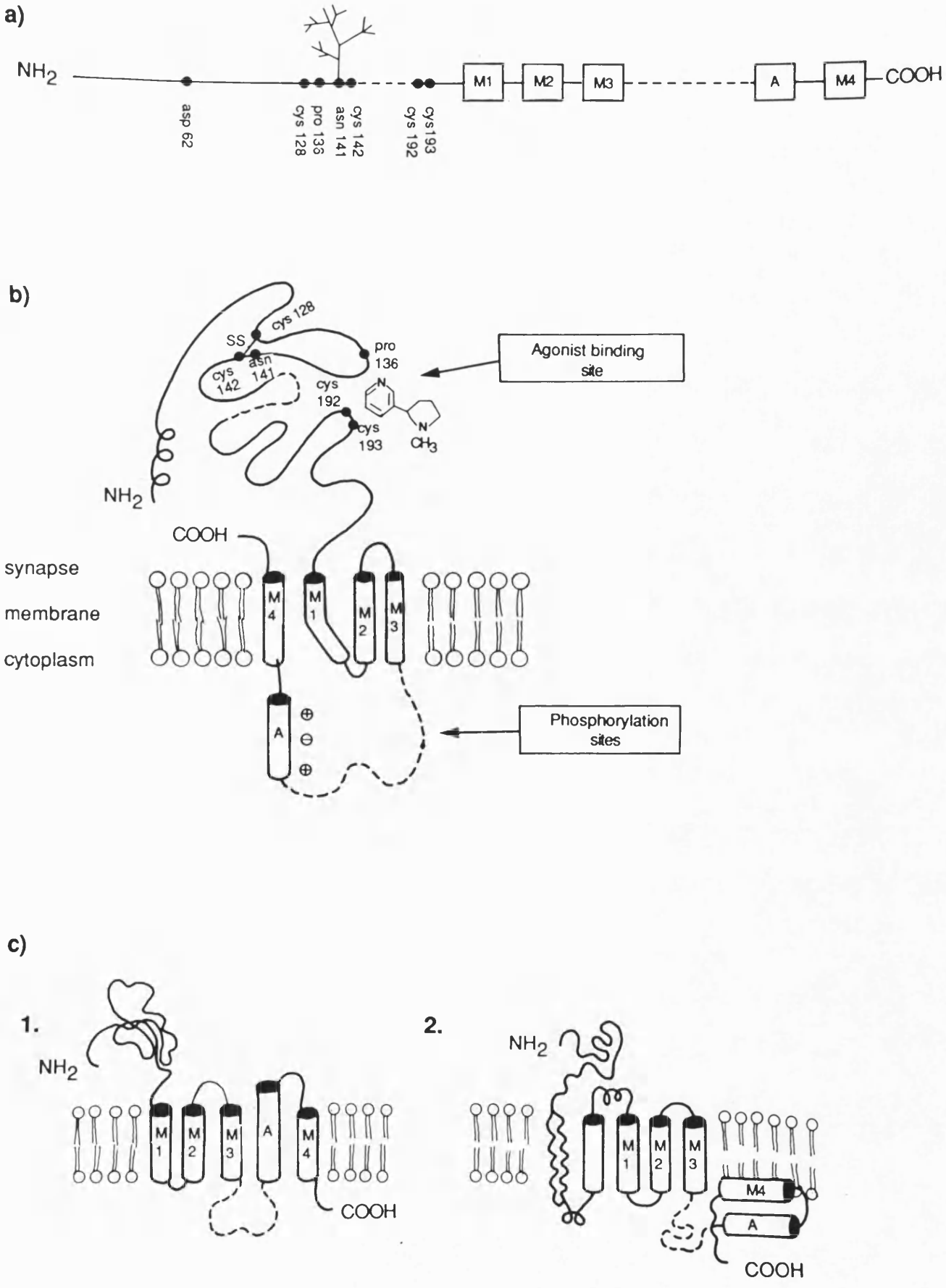
a) Primary sequence typical of nicotinic receptor subunits, showing the relative positions of the residues (note that cys 192 and 193 are only found in the α subunits), hydrophobic spans capable of membrane insertion (designated M1, M2, M3 and M4) and the amphipathic helix (A). Dashed lines indicate regions of variable length and low sequence conservation between subunits. Amino acid numbering is based on the Torpedo α subunit sequence.

b) Model of the transmembrane configuration of an α subunit, showing the relative positions of the conserved amino acids and domains indicated in (a). This simplified model is derived (V.B. Cockcroft, unpublished) from available biochemical and physicochemical data; the cytoplasmic and extracellular protrusions are consistent with the dimensions of the nicotinic receptor derived from electron imaging (see Fig. 3). The agonist binding site would face into the mouth of the receptor channel which is formed from 5 subunits creating a cylindrical structure. Four membrane crossings per subunit and an extracellular C-terminus have previously been proposed by Noda et al. (1982), Devillers-Thiery et al. (1983) and Claudio et al. (1983).

c) Alternative models propsed by 1. Guy (1984) and Finer-Moore and Stroud (1984), incorporating the amphipathic segment, A, as a membrane spaaning region, and 2. Ratnam et al. (1986).

Taken from Wonnacott (1989).

FIG. 4


a)
NH2
asp 62
cys 128
pro 136
asn 141
cys 142
cys 192
cys193
M1
M2
M3
A
M4
COOH
b)
SS
cys 128
pro 136
cys 142
asn 141
cys 192
cys 193
Agonist binding site
NH2
COOH
synapse
membrane
cytoplasm
M4
M1
M2
M3
A
Phosphorylation sites
c)
1.
NH2
M1
M2
M3
A
M4
COOH
2.
NH2
M1
M2
M3
M4
A
COOH

terminus is extracellular, the number of membrane crossings will determine whether the C-terminus is intracellular or extracellular. This, however remains in dispute.

The involvement of the amphipathic helix (A) in the formation of the putative ion-channel has been questioned. Recent evidence, based on photoaffinity labelling studies with non-competitive channel blockers (Giraudat et al., 1986; Hucho, 1986; Changeux and Revah, 1987; Changeux et al., 1987; Changeux et al., 1989) suggests that serine 262 of the δ subunit in M2 and its analogous residues in other subunits contributes to the ion-channel (Fig. 5a). It had previously been noticed (Guy, 1986) that the M2 region was the most highly conserved of all transmembrane regions. Expression studies involving chimeric δ subunits (Imoto et al., 1986), site-directed mutagenesis of specific clusters of amino acids in the serine 262 region (Imoto et al., 1988) and the construction of a synthetic peptide mimicking the sequence of the putative transmembrane M2 segment in Torpedo that forms discrete ion-channels in membrane bilayers (Oiki et al., 1988), have confirmed the importance of this M2 region in conductance and ion transport.

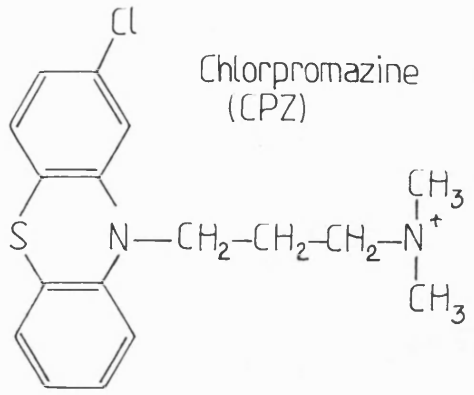
Fig. 5.

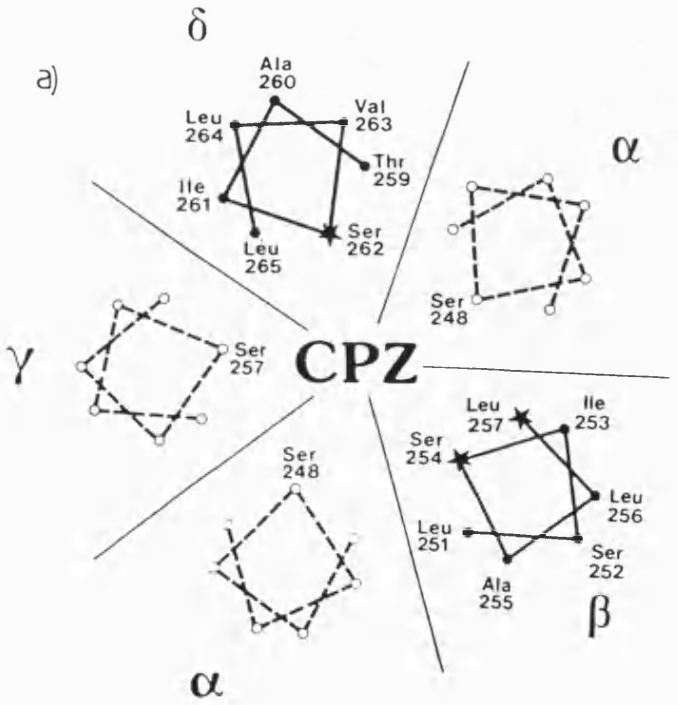
a) Hypothetical organisation of the ion channel based upon chlorpromazine (CPZ) labelling data: the transmembrane hydrophobic helix M2 is assumed to line the ion channel.

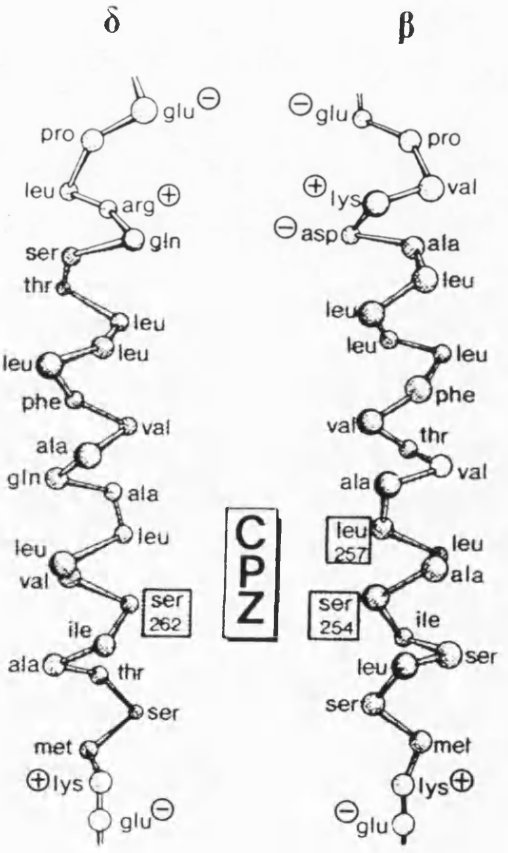
b) Hypothetical organisation of the acetylcholine binding site inferred from [P(N,N)-dimethylaminobenzene diazonium fluoroborate] (DDF) labelling data: according to this scheme three loops from the large hydrophilic domain would contribute to this site.

Taken from Changeux and Revah (1987).

FIG. 5


Cl
Chlorpromazine
(CPZ)
S
N—CH2—CH2—CH2—N+
CH3
CH3


a)
δ
α
γ
β
α
CPZ
Ala 260
Val 263
Thr 259
Ser 262
Leu 265
Ile 261
Leu 264
Ser 248
Ser 257
Ser 248
Leu 257
Ile 253
Ser 254
Leu 256
Leu 251
Ser 252
Ala 255


δ
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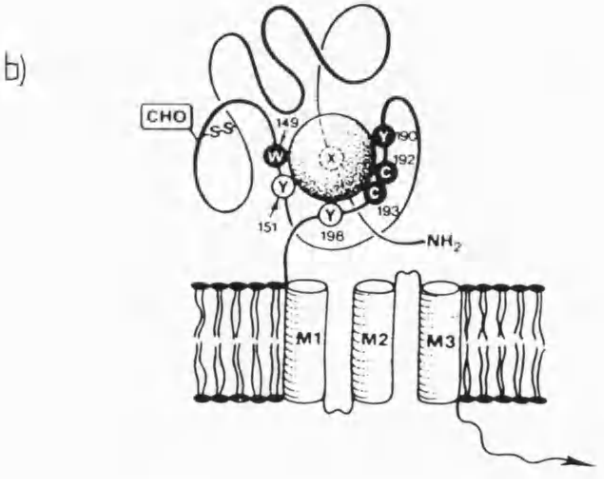

b)
CHO
SS
149
W
X
Y
190
192
C
C
Y
193
Y
151
198
NH2
M1
M2
M3

1.2.4.4. Acetylcholine binding site.

The binding sites for acetylcholine have been located, one on each of the synaptic surfaces of the two α subunits (Kistler et al., 1982; Kubalek et al., 1987). They can be labelled with α-BGT (Tzartos and Changeux, 1984; Wilson et al., 1984) or by the two major quarternary alkylating agents 4-(N-maleimido)-benzyltrimethylammonium (MBTA) (Weill et al., 1974; Cahill and Schmidt, 1984) and bromacetylcholine (Damle et al., 1978; Lydiatt et al., 1979). These affinity ligands are potent inhibitors of acetylcholine binding and covalently label the site by interaction with a reducible disulphide bond in close proximity to the acetylcholine binding site.

On the basis of the primary sequence of the α subunit, four cysteine (cys) residues were found in the N-terminal region at positions 128, 142, 192 and 193 (Noda et al., 1982; Devillers-Thiery et al., 1983). It was concluded that a reducible disulphide bond existed between cys 128 and 142. Using the affinity labelling technique, Kao and Karlin (1984) demonstrated that MBTA labelled the adjacent cys residues at positions 192/193 and not at 128 and 142. As homologues of cys 128 and 142 occur in all subunits but cys 192/193 are unique to the α subunit (Noda et al., 1983a; Claudio et al., 1983), the functional roles of each of these cys residues in the α subunit was investigated by replacing each cysteine with serine by site directed mutagenesis

(Mishina et al., 1985). From their results, Mishina et al. (1985) speculated that cys 192/193 of the α subunit have a specific role in agonist binding, whilst cys 128 and 142 are essential for maintaining the proper conformation of the AChR molecule; they did not, however, rule out the possibility that these residues might also be involved in agonist binding (see also Fig. 5b).

1.2.5. Immunological profile of AChR.

Antibodies made in animals immunised with native AChRs are directed primarily at the extracellular surface of the AChR (Froehner, 1981). Although antibodies are produced to many parts of the AChR, 60-70% are directed at one region found on the α subunits (Tzartos and Lindstrom, 1980; Tzartos et al., 1981,1983). Monoclonal antibodies (mAbs) have been mapped on native receptor using competitive binding techniques (Tzartos and Lindstrom, 1980; Tzartos et al., 1981). This approach showed that the majority of mAbs raised against an intact receptor were directed at the same particular region, termed the main immunogenic region (MIR). The binding sites for some of the mAbs could be estimated with some precision after the elucidation of the primary sequence (see section 1.2.4.1.) and the MIR has been mapped to the general region between amino acids 46 and 127 on the α subunit

(Ratnam et al., 1986; Barkas et al., 1987; Kubalek et al., 1987). The MIR is a well conserved feature of the AChR, but its function is unknown (Lindstrom et al., 1988), although it is known to be important in the autoimmune disease myasthenia gravis.

1.2.6. Neuronal AChR.

In the central nervous system, there are nicotinic AChRs which belong to the same gene family as AChRs from muscle (for review, see Lindstrom et al., 1987). These AChRs can, however, be distinguished from muscle AChRs by virtue of their pharmacological profile (for reviews, see Wonnacott, 1987; Colquhoun et al., 1987).

AChRs found at the ganglia have μM affinity for nicotine, as do muscle AChRs (Kemp and Morley, 1986), whereas AChRs from brain have nM affinity for nicotine. Unlike muscle AChRs, the neuronal AChRs do not bind α-BGT (Lindstrom et al., 1987).

In comparison with the muscle AChRs, neuronal AChRs have only two types of subunit, one is believed to be structural like the β, τ or δ subunits from muscle AChRs, the other is acetylcholine binding, like the α subunits from muscle (Whiting and Lindstrom, 1987). In neurons, there are also α-BGT binding proteins of unknown function and uncertain composition. These are members of the AChR gene family but do not contain the AChR gated cation channels (Lindstrom et al., 1987).

1.3. Myasthenia gravis.

The first evidence for the direct implication of the neuromuscular junction in MG came after Mary Walker (1934) noticed similarities in patients with MG and patients suffering from curare poisoning.

The elucidation of the precise site of the defect in MG was aided by electrophysiological studies. The muscles of MG patients have abnormally small end plate potentials (epps), which are either below the threshold required to initiate the contractile processes or rapidly become so during repetitive stimulation (Elmqvist et al., 1964). Other studies revealed that in MG patients the action potential generated by the nerve was normal but that the miniature end plate potentials (mepps) were reduced (Elmqvist et al., 1964; Santa et al., 1972; Albuquerque et al., 1976), while in vitro studies showed that motor end-plates showed reduced sensitivity to iontophoretically applied acetylcholine (Rash et al., 1976). The number of acetylcholine molecules per quantum (Cull-Candy et al., 1979) and the number of quanta per impulse (Elmqvist et al.,1964; Lindstrom and Lambert, 1978; Cull-Candy et al., 1978, 1980) have now been shown to be normal at myasthenic nerve terminals, reinforcing the idea of a post synaptic defect. The question of the location of the primary lesion was, in fact, resolved following the availability

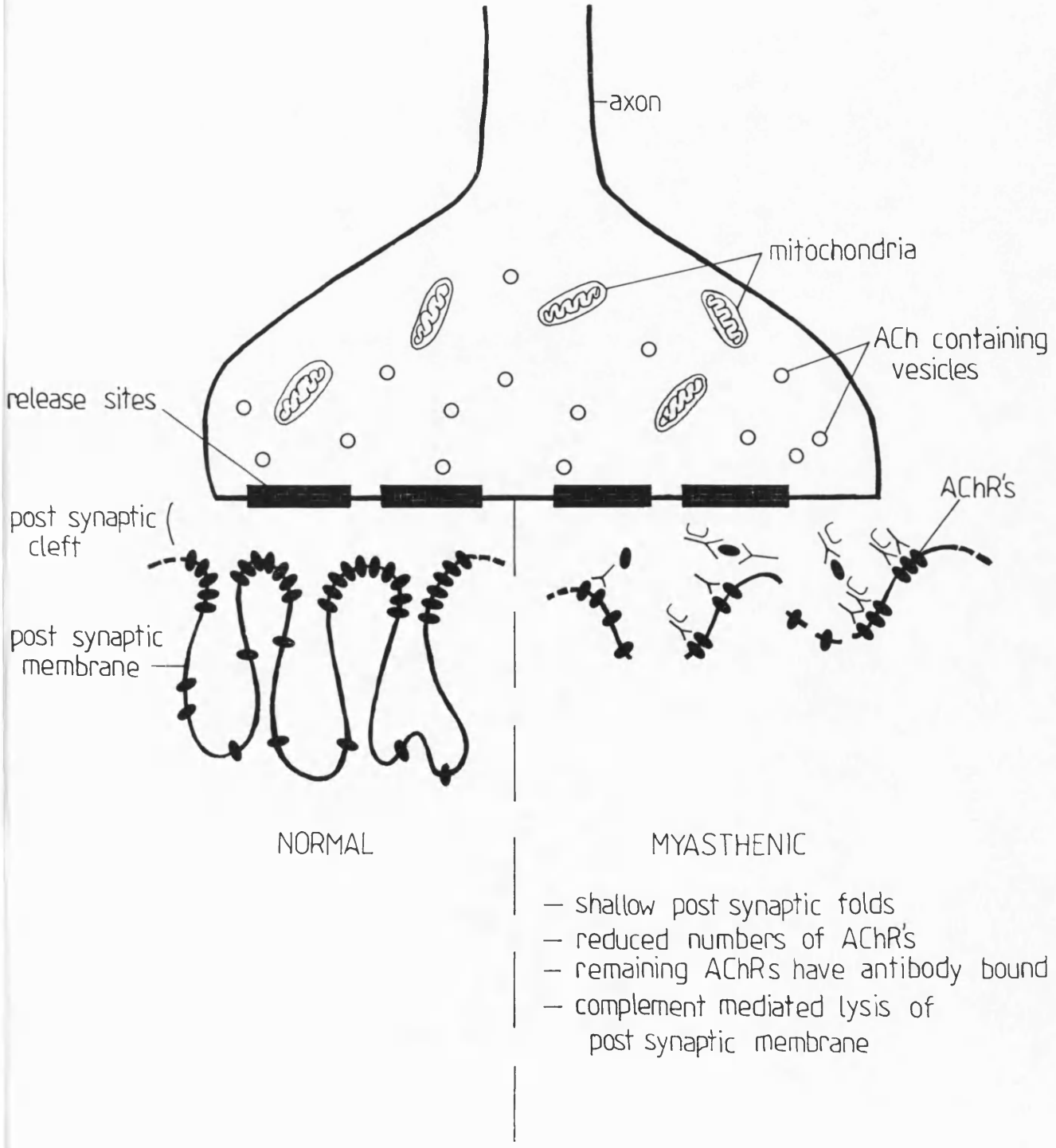
of the post-synaptically acting snake neurotoxins (Section 1.3.2.). Using iodinated α-BGT, Fambrough et al. (1973) demonstrated a reduction in the number of α-BGT binding sites in myasthenic end-plates, indicating a reduced number of AChRs. It has been experimentally demonstrated that a reduction in the number of AChRs is sufficient to produce the characteristic features of MG.

Changes in the morphology at myasthenic end-plates suggest degeneration and regeneration of the neuromuscular junction with flattening and simplification of the post synaptic folds (fig. 6). The reduction of AChRs and the morphological changes observed in myasthenic end-plates compromise neuromuscular transmission. If this is reduced to the extent that the threshold value is not reached, action potentials will not be triggered. This will ultimately lead to the reduction of muscle power leading to muscle weakness and susceptibility to fatigue that is characteristic of MG.

1.3.1. Autoimmune nature of MG.

The autoimmune nature of MG was first suggested by Smithers (1959) on the basis of histological similarities between the thymus gland in MG patients and the thyroid of patients with Hashimoto's Thyroiditis. Simpson (1960) had also noticed associations between MG and known autoimmnune diseases and postulated that MG

FIG. 6 Comparison of normal and myasthenic neuromuscular junctions



was caused by antibodies to the receptive substance at the motor end-plate. Other immunological features relating to MG were also discovered: Nastuk and co-workers observed wide variations in serum complement levels in MG patients (Nastuk *et al.*, 1960), while Strauss *et al.*(1960) reported the presence of serum complement-fixing antibody bound *in vitro* to skeletal muscle sections. The latter study was the first demonstration of a circulating tissue-specific antibody related to MG. These antibodies were later shown to be connected to a tumour of the thymus, however, and not specific to MG (Oosterhuis *et al.*, 1976; Limburg *et al.* 1983).

That the AChR is the target of the autoimmune attack in MG was indicated by the serendipitous experiment of Patrick and Lindstrom (1973), who immunised rabbits with purified piscine AChR and observed severe myasthenia-like symptoms in the animal. This experiment, coupled with those of other workers (Sugiyama *et al.*, 1973; Heilbronn and Mattson, 1974) provided an experimental model (experimental autoimmune myasthenia gravis, EAMG) for further study of the human disease.

Antibodies to skeletal muscle AChRs, were found after Almon *et al.* (1974) demonstrated that approximately 50% of myasthenic sera tested could inhibit α-BGT binding to rat denervated preparations. The serum factor was later identified as IgG (Almon and Appel, 1975). Bender *et al.* (1975), using indirect immunoperoxidase techniques, showed that myasthenic sera

blocked the binding of α-BGT to motor end-plates of normal human muscle. The ability of a high proportion of myasthenic serum antibodies to fix complement in the presence of small quantity of Torpedo AChR was also demonstrated (Aharanov et al. 1975).

A radioimmunoassay for the quantitation of anti-AChR antibodies in MG sera soon became a standard diagnostic procedure (Lindstrom et al., 1976; Lindstrom, 1977; Monnier and Fulpius, 1977; Newsom-Davis et al., 1978; Dwyer et al.,1979; Tindall et al., 1981; Carter et al., 1981). This procedure uses crude detergent extracts of human limb muscle as the source of AChR antigen. The detection of positive serum anti-AChR antibody levels is specific for the diagnosis of MG and approximately 90% of patients have elevated levels relative to controls (Lindstrom et al., 1976; Lindstrom, 1977; Mittag et al., 1981, 1984; Monnier and Fulpius, 1977; Ito et al., 1978; Lefvert et al., 1978).

1.3.2. Anti-AChR antibody characteristics.

The anti-AChR antibodies found in MG sera are predominantly of the IgG fraction (Rodgaard, et al., 1987), although antibodies of the IgM class have been reported (Lefvert et al., 1978). In individual MG patients, anti-AChR antibodies of different IgG subclasses have been identified (Vincent and Newsom-Davis, 1980; Lefvert et al., 1981; Tindall, 1981,

Whiting et al., 1983; Rodgaard et al., 1987). A small proportion of anti-AChR antibodies in MG are directed against the α-BGT binding site (Dwyer et al., 1979; Mittag et al., 1981; Vincent and Newsom-Davis, 1979, 1980, 1982; Whiting et al., 1983).

These findings indicate that the antibodies are polyclonal, being directed to more than one determinant on the AChR of skeletal muscle. Monoclonal antibodies have been produced which bind to a variety of sites on the AChRs (Tzartos and Lindstrom, 1980). Most, however, bind to a region on the α subunit distinct from the cholinergic binding site, known as the main immunogenic region (MIR) (see Section 1.2.5.), and the use of such monoclonals in competitive binding studies has shown that the majority of human anti-AChR antibodies are directed at the MIR. Some antibodies are directed at other sites on other subunits and only a few are directed at non-MIR regions on the α subunit (Tzartos et al., 1982, 1983).

The heterogeneity of the antibodies in MG most probably helps to explain the well documented lack of correlation between clinical status and serum anti- AChR antibody titres (Lindstrom et al., 1976; Ito et al., 1978; Lefvert et al., 1978).

1.3.3. Pathogenicity of anti-AChR antibodies.

It is generally accepted that depletion of AChR

constitutes the major factor leading to impaired neuromuscular transmission in MG, and that anti-AChR antibodies play an important role in this depletion. Whether the circulating anti-AChR antibodies are pathogenic, or represent a secondary response to damage caused by some other agent, is important in understanding the pathogenesis of MG.

The following observations support the idea that humoral factors are the primary agents in the disease process;-

i) Plasmaphoresis of myasthenic patients is associated with temporary clinical improvement, paralleled by a decrease in the concentration of serum antibodies. (Pinching et al., 1976; Dau et al., 1977; Newsom-Davis et al., 1978).

ii) Placental transfer of anti-AChR antibodies from myasthenic mother to fetus can cause transient neonatal MG, which remits as the titre declines. (Keesey et al., 1977).

iii) Injection of mice with myasthenic IgG produces the clinical symptoms of the disease associated with a reduction of mepps and α-BGT binding (Toyka et al., 1977).

If the anti-AChR antibodies are to cause synaptic dysfunction, they are required to leave the vascular system, diffuse into the extracellular space, enter the synaptic cleft and reach the AChRs on the synaptic

folds. Detection of IgG at the motor end-plates in MG patients was achieved in an elegant study, by Engel et al. (1977) using peroxidase-labelled Staphylococcal Protein-A. These facts support the idea that the serum antibodies are one of the primary agents in the disease process.

The mechanisms by which this might be achieved are:

a) Direct immunopharmacological blockade of acetylcholine binding.

b) Accelerated degradation of AChR.

c) Lysis of the post-synaptic muscle membrane, possibly enhanced by complement.

The first two mechanisms have been extensively studied (for reviews see Vincent, 1980; Harrison and Behan, 1986), but there has been little investigation of the third mechanism.

1.3.4. Complement.

Complement is a series of nine protein components (1-9), present in the serum in an inactive form (for reviews see Alexander and Good, 1977; Lachmann and Peters, 1982). Several of the components are pro-enzymes, awaiting the activation of their predecessor in the sequence to convert them into their active form, frequently by a proteolytic step. All the components initiate effector mechanisms which can produce a rapid amplified response to a trigger stimulus.

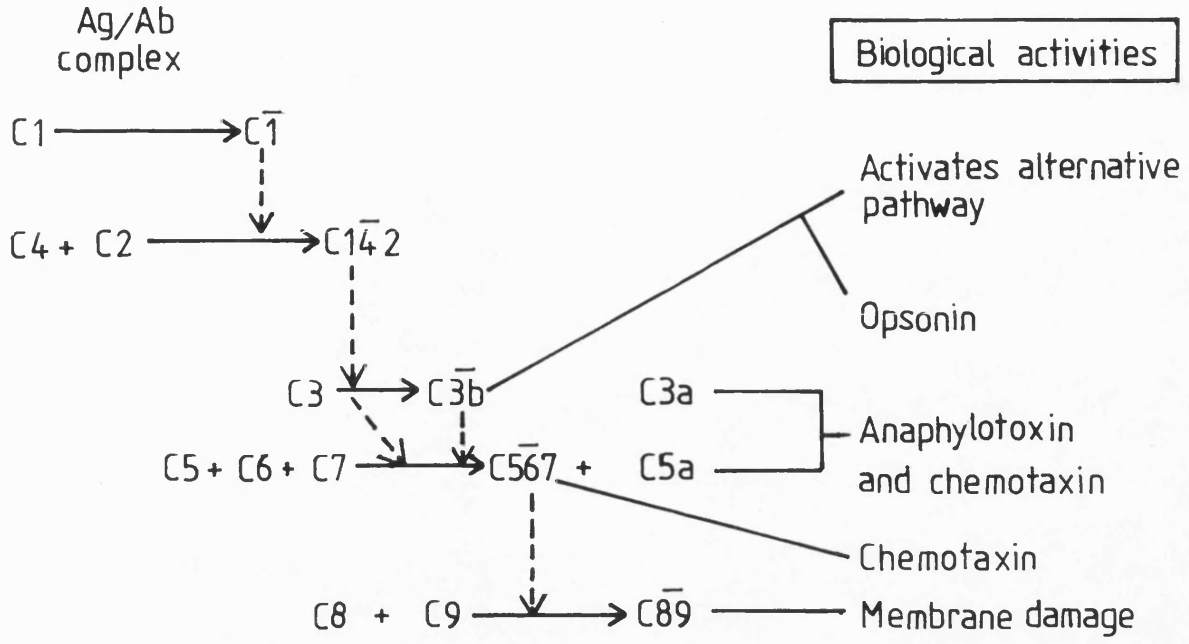
There are two main pathways of complement

activation (see Fig. 7). The classical pathway, which is initiated by C1q (one of the components of C1) binding to antigen-antibody complexes, and the alternative pathway, which is initiated in the absence of antibody by such stimuli as zymosan a yeast cell wall polysaccharide, cobra venom factor and properdin. When C9, the terminal component of the complement pathway is activated by the classical or alternative pathways, transmembrane channels are produced in susceptible membranes. These channels permit loss of intracellular constituents, entrance of extracellular ions and osmotic lysis of the cell or membrane bearing the complex. Fig. 8 shows a diagrammatic representation of the mechanism of action of complement in MG. The complement system plays an important role in inflammatory and immune reactions, and in the destruction of cells and cell membranes via complement-mediated lysis.

1.3.5. Involvement of complement in MG.

Involvement of complement in MG was first suggested by Nastuk *et al*. (1960), who noticed that serum complement levels fell during exacerbation of the disease and proposed that complement was bound to antigen-antibody complexes on myasthenic end-plates, thereby reducing levels in the serum. This observation followed an earlier study by Nastuk *et al*. (1959) who

FIG. 7 a) Classical pathway of complement activation

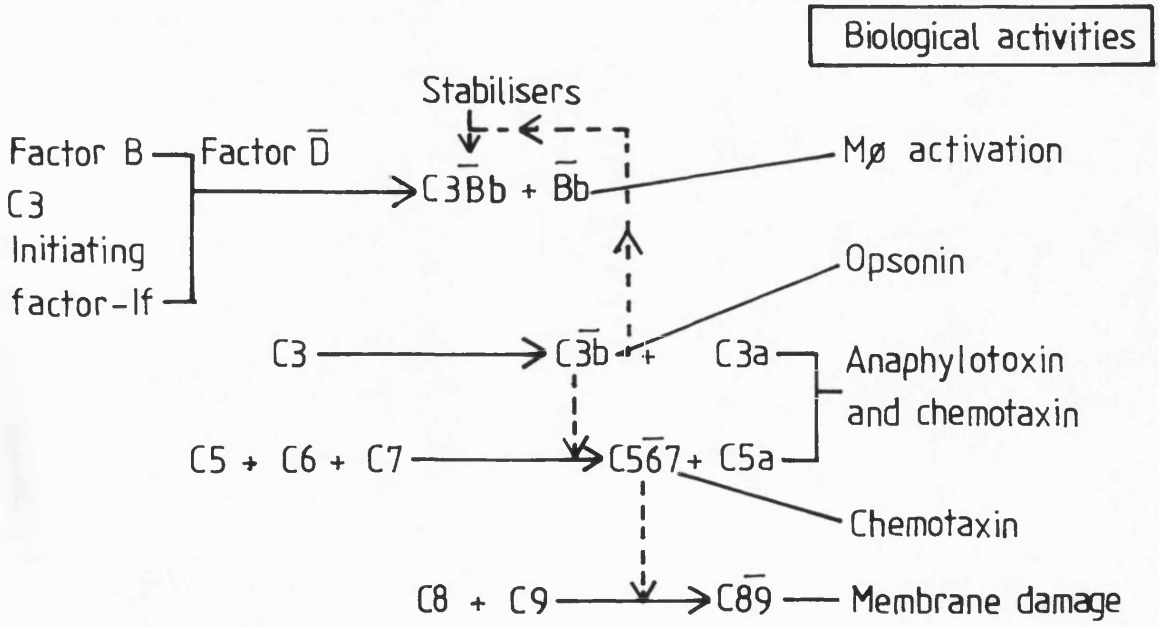


Key

C N native complement factor

C N̄ activated complement factor

b) Alternative pathway of complement activation

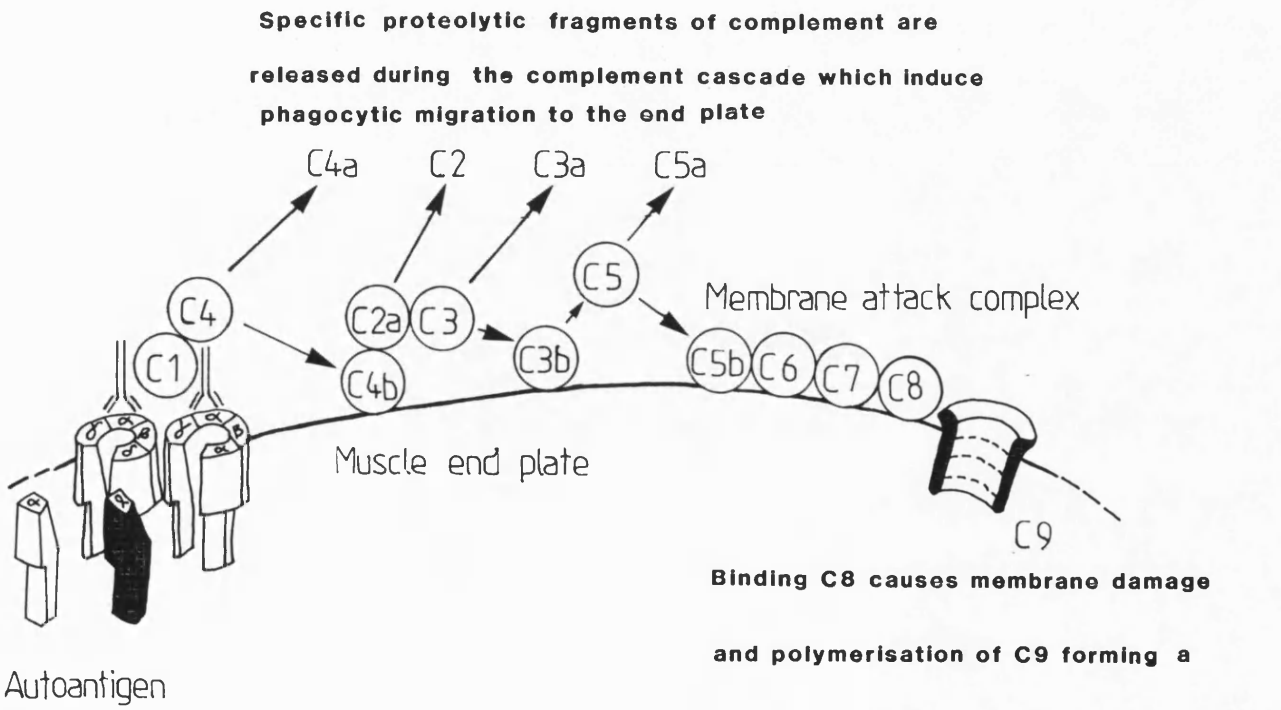


Key

Factor B native factor

Factor B̄ activated factor

FIG. 8 Proposed mechanism of complement in myasthenia gravis



found that 2 out of 22 myasthenic serum samples were cytolytic to frog muscle in vitro, although control sera showed some cytolytic activity after a longer incubation period. Liveson et al. (1976) also noticed a cytolytic effect of myasthenic sera on mouse muscle cells in culture and its abolition by heating the sera (destroying any complement activity). However, other studies of this nature failed to demonstrate a complement mediated lytic effect (Harvey et al. 1978) even when IgG and C3 were demonstrated by immunofluorescence to be bound to the cells (Lennon, 1978).

Ultrastructural and light microscopic studies by Engel et al. (1977) using peroxidase labelled Staphylococcal Protein-A and rabbit anti-(human-C3) antiserum, showed the presence of IgG and C3 on the muscle end-plates of myasthenic patients. In a continuation of these studies (Sahashi et al., 1980) showed the presence of C9 on the muscle end-plates and of IgG, C3 and C9 on disintegrating junctional folds and on the debris in the synaptic cleft. These studies provided the first evidence for antibody dependent complement-mediated injury to the post-synaptic membrane in MG. Using similar techniques, Engel and Arahata (1987) have more recently demonstrated the presence of the MAC at the end plates of seven myasthenic patients. The latter evidence is probably the strongest with regard to the simplification and remodelling of the post-synaptic membrane in MG (see Fig. 6).

The involvement of C3 in EAMG, passively transferred to mice by injection of myasthenic IgG was investigated by Toyka et al., (1977). Animals first treated with cobra venom factor to deplete C3, showed a reduction in mepp amplitudes and α-BGT binding to muscle end-plates was less marked than in untreated animals. C3 depletion was also found to minimise the effects of passive transfer of EAMG by myasthenic serum to rats (Howard and Sanders, 1980). C5 was inferred not to be involved in EAMG, as genetically C5 deficient mice reacted similarly to non C5 deficient controls when injected with myasthenic IgG (Toyka et al., 1977).

Direct experimental evidence of complement-mediated lysis in the human disease is lacking. A major reason for this probably lies in the difficulty in identifying and quantifying muscle cell lysis.

It is known that skeletal muscle cells, in culture, express a surface AChR, the appearance of which coincides with the fusion of myoblasts to form myotubes. Such cultures have been used to investigate complement mediated damage, evidence for which which has depended on either a visual assessment or release of radiolabelled chromium. Both methods have been criticised; the first can only detect gross disruption and the second lacks specificity, in that radiolabelled chromium is taken up not only by the cultured myotubes, but also by the fibroblasts which accompany them in culture. The proportion of fibroblasts can be reduced by the use of cytotoxic drugs but they cannot be completely

eliminated.

Cambridge and Stern (1981) published a procedure for measuring myotube specific cytotoxicity based on the use of radiolabelled carnitine. The method was designed to assay the myotoxicity of lymphocytes from patients with polymyositis, and depends on the selective uptake by cultured myotubes of tritiated carnitine. The subsequent loss of radioactivity can be taken as a measure of cell lysis. This procedure is less sensitive to the presence of fibroblasts, which take up carnitine five times slower than do myotubes (Cambridge and Stern, 1981).

This assay was used to advantage to demonstrate lysis, by myasthenic serum and complement, of chick myotubes (Bird, 1986) and of cultured rat myotubes (Childs et al., 1984, 1985, 1987). Ashizawa and Appel (1985) confirmed these results in a similar release assay, using radiolabelled rubidium instead of carnitine. A preliminary study (Childs et al., 1985) showed that the lytic property of serum was lost if anti-AChR antibodies were removed on an antibody affinity column.

These results complement the ultrastructural studies of Engel and colleagues in indicating the probable involvement of complement-mediated lysis as a factor in the myasthenic disease process. A summary of reports concerning the involvement of complement in the pathogenesis of MG is given in Table 1.

Table 1. Involvement of complement in MG and EAMG.

Involvement of complement	Reference
Cytolytic properties of MG serum towards frog muscle *in vitro*.	Nastuk *et al*. (1959)
Reduction in serum complement levels during exacerbation of disease.	Nastuk *et al*. (1960)
Cytolytic properties of MG serum towards mouse muscle cells *in vitro*.	Liveson *et al*. (1976)
Light microscopic demonstration of IgG and C3 on myasthenic muscle end plates.	Engel *et al*. (1977)
Reduction in mepp amplitudes and α-BGT binding after depletion of C3.	Toyka *et al*. (1977)
Immunofluorescent detection of IgG and C3 on cultured rat muscle cells.	Lennon, (1978)
Light microscopic demonstration of IgG and C3 on intact junctional folds of Lewis rats injected with IgG from EAMG rats.	Engel *et al*. (1979)
C3 depletion found to minimise passive transfer of EAMG to rats.	Howard and Sanders (1980)
Light microscopic demonstration of IgG, C3 and C9 on disintegrating junctional folds.	Sahashi *et al*. (1980)
Complement mediated lysis of rat muscle cells *in vitro*.	Childs *et al*. (1984, 1985,1987)
Complement mediated lysis of rat muscle cells *in vitro*.	Ashizawa and Appel (1985)
Complement mediated lysis of chick muscle cells *in vitro*.	Bird (1986)
Ultrastructural localisation of MAC in myasthenic patients.	Engel and Arahata (1987)

1.3.6. Cellular involvement in MG.

The mechanisms that trigger the sensitisation to AChR and subsequently maintain the levels of circulating anti-AChR antibodies are unknown. As in other autoimmune diseases, the immune system in MG is responding to a "self-antigen" in an inappropriate way. This could result from:-

1) An alteration in the antigen, causing it to be perceived as "non-self".

2) Abnormal regulation of the immune system, allowing it to respond to a normal antigen.

3) An exogenous antigen that resembles the relevant autoantigen and thus triggers an autoimmune response.

In MG, it is possible that the immune mechanisms involved are not always the same in different patients, although the target of the autoimmune attack is probably always the AChR.

The malfunction in MG may originate in the thymus gland. Castleman (1966), drew attention to a high incidence of thymoma and other pathological changes in the thymus of patients with MG. The thymus is responsible for the development of immunocompetent T-cells, which are involved in both regulatory and effector functions. Thymic myoid cells are known to express AChR (Wekerle et al., 1975; Kao and Drachman, 1977) and may provide the initiating stimulus for

lymphocyte production of anti-AChR antibodies. The recognition of thymic AChR as "non-self" could occur as a consequence of 1 or 2 listed above.

Thymic cells from patients with MG can spontaneously synthesise anti-AChR antibody in culture (Vincent et al., 1978) and are able to enhance anti-AChR antibody production by autologous blood lymphocytes (Newsom-Davis et al., 1981). Willcox et al. (1984) suggested that this enhancement could be mediated by antigen-presenting cells rather than by AChR-specific T-helper cells in the thymus.

It has been suggested that the relative proportions of T-suppressor and T-helper cells, which regulate antibody synthesis by B-lymphocytes, could be important in MG. Studies using monoclonal antibodies and indirect immunofluorescence as phenotypic markers for T-cells have concluded that there are decreased numbers of circulating suppressor cells in MG (Bahir et al., 1981; Skolnik et al., 1982). Decreased suppressor cell activity has also been inferred from the impaired ability of myasthenic T-cells compared to normal controls, to inhibit anti-AChR antibody production by myasthenic lymphocytes (Shinomiya and Yata, 1981). The autologous mixed lymphocyte reaction (AMLR) involves the proliferation of T lymphocytes when co-cultured with autologous non-T cells and may reflect *in vivo* mechanisms of immune control. An investigation by Richards et al. (1986), found that the AMLR in myasthenic patients was significantly depressed compared

to normal controls. The proliferative responses of T-cells from myasthenic patients to the mitogens Conconavalin-A and Phytohaemagglutinin-P were also found to be reduced. These results were, in part, interpreted in terms of defective suppressor cell functions in MG (Richards et al., 1986). In general, however, studies on specific T-cell populations in MG have proved inconsistent (for reviews, see Lisak et al., 1985; Harrison and Behan, 1986).

There are several reports of increased in vitro proliferative responses by myasthenic peripheral blood lymphocytes to purified electric fish or human AChR (Abramsky et al., 1975; Richman et al., 1976; Conti-Tronconi et al., 1977; McQuillen et al., 1983; Hohlfield et al., 1984; Nickless, 1985) and fetal calf AChR (Nickless, 1985).

Early morphological studies reported the presence of lymphorrhages (collections of lymphocytes), surrounding necrotic muscle fibres in approximately 20% of myasthenic muscle biopsies (Engel and McFarlin, 1966, Woolf, 1966). However, there is no evidence that cell-mediated immune mechanisms are directly responsible for the altered end-plate morphology seen in MG. It is probable that the enhanced proliferative response in vitro is indicative of an increased number and/or activity of specific helper T-cells which interact with antigen presenting cells and B-cells to produce anti-AChR antibodies (Lisak et al., 1985)

1.4. Tissue and cell culture.

The study of the development and function of the muscular system must at some point focus on the development and function of the individual cells. Although this is difficult to investigate *in vivo*, tissue culture permits the study of the individual cell types in an environment that minimises the multiple interactive events characteristic in the animal. Purified populations of muscle cells, or homogeneous populations derived from clonal cell lines, have provided preparations suitable for biochemical and electrophysiological analysis.

1.4.1. Muscle culture.

The discovery that explanted fragments of frog embryo nervous tissue survived in the presence of clotted lymph (Harrison, 1907) was a major breakthrough in tissue culture. These experiments not only provided the first use of tissue culture for the study of the nervous system, but also exemplified the potential inherent in tissue culture. Harrison's techniques were subsequently adapted to include tissue from other species. Lewis (1915) described the behaviour of explants of chick embryo leg muscle in culture. Some outgrowths of this culture demonstrated spontaneous

contractility in the absence of nerves. After the establishment of enzymic dissociation techniques (Moscona, 1952; Rinaldini, 1959), studies were made on embryonic skeletal muscle grown in monolayer cultures and the use of skeletal muscle cultures in cell biology and biochemistry has since flourished.

The use of dissociation techniques to establish monolayer muscle cultures offers several advantages over previous explant tissue culture type investigations. The cells grow and develop in a synchronous manner and such cultures are particularly appropriate for the biochemical analysis of myogenesis. The cells can be maintained under uniformly controlled conditions and may be subjected to a wide range of experimental interventions. Successful cultures have been prepared from skeletal muscles of several species, including chick, mouse, rat and human (for reviews, see Hauschka, 1972; Yaffe, 1973; Konigsberg, 1979). Further work has concerned methods for growing human muscle in dissociated cell cultures (Yasin et al., 1977; Blau and Webster, 1981). One of the main attractions of applying tissue culture methods to skeletal muscle is the potential of using human tissue to investigate muscular and muscle-related diseases.

1.4.2. Development of acetylcholine receptors.

Studies of embryonic muscle in vitro have demonstrated that many properties of differentiated

muscle in vivo are reproduced in cell cultures. In both cases, a proliferating pool of mononucleated cells is the precursor of multinucleated muscle fibres (Konigsberg, 1963; Yaffe, 1969).

Using tissue culture, it has been possible to study the development of acetylcholine sensitivity in skeletal muscle and the mechanisms controlling the production and distribution of AChRs. The fusion of myoblasts and the formation of multinucleated cells is the biochemical signal that triggers the production of many muscle specific proteins including cholinoceptors. In support of this, many workers have reported little or no response to iontophoretically applied acetylcholine in undifferentiated, uninucleated cells in culture (Dryden, 1970; Famborough and Rash, 1971; Harvey et al., 1979), sensitivity appearing only after the formation of multinucleated cells. However, binding of α-neurotoxins (Patrick et al., 1972; Prives and Patterson, 1974) and autoradiography (Powell and Famborough, 1973; Sytkowski et al., 1973; Blau and Webster, 1981) have revealed low levels of AChRs on myoblasts, increasing rapidly after fusion. Moreover, a small population of myoblasts do differentiate to become sensitive to acetylcholine (Famborough and Rash, 1971; Dryden et al., 1974) and this process can be increased by blocking fusion with a low Ca++ medium (Famborough and Rash, 1971).

On young myotubes, acetylcholine sensitivity and toxin binding sites are uniformly distributed. On further development, areas of high sensitivity and toxin

binding can be detected (Vogel *et al.*, 1972), with clusters of binding sites (hot spots) increasing as myotube development proceeds (Sytkowski *et al.*, 1973). However, with increasing age in culture, there is a gradual disappearance of receptor clusters (Axelrod *et al.*, 1976: Prives *et al.*, 1976) ascribed to an effect of muscle contractility.

1.4.3. Cell Lines.

Cell lines are particularly convenient as experimental models, providing consistent and reproducible systems.

In order to exploit clonal cell lines for the study of biological problems, a collection of cells is required that is representative of the *in vivo* cell types. A number of cell lines have been described, some obtained by selectively cultivating tissue samples [eg: the clonal cell line, L6 (Yaffe, 1968); the mouse C2 cell line (Yaffe and Saxel, 1977); the BC3H1 cell line (Schubert *et al.*, 1970)] and others derived from spontaneous or chemically induced neoplasms [PC12 cells (Greene and Tischler, 1976), IMR32 cells (Tumilowicz *et al.*, 1970) and TE671 cells (McAllister *et al.*, 1977)].

1.4.4. TE671 cell line.

The human medulloblastoma clonal cell line TE671,

was thought to be derived from a primitive neuroectodermal tumour of the cerebellum, sharing neural tube origins with other cells of the central nervous system (McAllister et al., 1977). Cerebellar medulloblastoma is a rare central nervous system tumour type made up of cells that express a modicum of neuronal properties (Rorke, 1983). However, more recent findings dispute the neuronal origin of the cells (see Section 1.13.4.8.).

1.4.4.1. Cellular morphology.

There is evidence that the cell line is polymorphic in culture, exhibiting 5 (Syapin et al., 1982), or 6 (Zeltzer et al., 1984) morphological cell types (Table 2). All cell types are present at all stages of culture in varying proportions (Syapin et al., 1982), although as the cells reach confluence, morphological definition becomes less apparent. It has been suggested (Lukas, 1986a) that transitions in TE671 cellular morphology are a consequence of the passage of individual cells through the replication sequence.

1.4.4.2. Pharmacological profile.

Syapin et al. (1982) established that all cell types were capable of generating Na+ dependent action potentials and further demonstrated that four out of the five cell types showed changes in conductance as a

Table 2. Morphological types of TE671 cells.

Cell	Syapin et al. (1982)	Zeltzer et al. (1984)
1	Large, flat, sometimes multinucleated cells.	Large multinucleated cell.
2	Small round or polygonal cells.	Circular or round shaped cells.
3	Long centripolar bipolar spindle	Bipolar or tri polar shaped cells.
4	Monopolar, mononuclear cells.	Large mononuclear cell.
5	Small flat cells sometimes with branching extensions.	Spindle shaped cell.
6	-	Giant multinuclear cell.

response to iontophoretically applied acetylcholine but not to τ-aminobutyric acid (GABA), indicating the presence of functional AChRs. Fluorescent labelling studies (Luther et al. (1989) have also indicated that AChRs are present on the surface of some cells, although these experiments were carried out on serum starved TE671 cell cultures and it is not known what effect serum starvation may have on AChR expression.

The acetylcholine induced depolarising response was found to be inhibited by low concentrations of nicotinic antagonists, α-BGT (10^{-6}M) and d-tubocurarine (10^{-5}M) and only by high concentrations of the muscarinic antagonist, atropine (Syapin et al., 1982). The fact that the depolarising response in TE671 cells is blocked by low concentrations of α-BGT, contrasts with the ineffectiveness of α-BGT in other cultured mammalian neuronal cell types (Patrick and Stallcup,1977).

Owing to the fact that the AChR family includes muscle type AChRs, neuronal AChRs and neuronal α-BGT binding proteins (see Section 1.2.6.), all of which exhibit related but distinct biochemical properties, the TE671 cell AChRs were initially classified as neuronal AChR types which could be blocked by α-BGT (Syapin et al., 1982). This was clearly unusual and stimulated further investigation.

Lukas (1986abc) noted that [^{125}I] α-BGT binding sites on TE671 cells were unique in neuronal terms, bearing more similarity to sites on rat muscle or Torpedo electroplax than to those of PC12 cells or rat

brain. These findings accord with those of de la Garza et al. (1987), who showed α-BGT to be effective in antagonising cerebellar interneuron excitation by nicotine, in an essentially irreversible manner, suggesting that nicotine action in the cerebellum may be mediated by receptors similar to those at the neuromuscular junction.

A pharmacological similarity of TE671 cell AChR and that of muscle (human calf) was also noticed by Walker et al. (1988). These workers noted, in addition, that properties of AChRs in total TE671 cell extracts may be different from those present on the surface of the cells. Varying sensitivities to cholinergic antagonists were observed, which may reflect the stage of confluence of the cells (Lukas, 1986a).

1.4.4.3. Immunological reactivity.

Immunological reactivity was investigated, initially by Lukas (1986abc), using the rationale that antibodies raised against AChR from electric organ or muscle might recognise highly conserved epitopes and so act as probes for a neuronal AChR. Immunological reactivity was further investigated by Lindstrom et al. (1987) and by Whiting et al. (1987a), who found that the TE671 cell AChR bound antisera and monoclonal antibodies raised against both Torpedo and muscle and also that extracts of TE671 cell AChRs could bind serum from patients with myasthenia gravis containing anti-AChR antibodies.

Lindstrom et al. (1987) also reported that extracts of TE671 cells could be used in the immunoprecipitation assay for anti-AChR antibody. The usefulness of the TE671 cell AChR as a reproducible source of antigen for this assay in myasthenia gravis has more recently been investigated by Walker et al. (1988), who found that the total number of binding sites available in TE671 cell extracts is less than that found in many calf muscle preparations, and concluded that the cell line offers no advantage as a source of antigen for the immunoprecipitation assay.

These data, overall, suggest that the AChR of TE671 cell is similar or even identical to that of skeletal muscle, differences arising between the two receptors from post-translational modifications or from the vagaries of proteolysis (Whiting et al., 1987a).

1.4.4.4. Electrophysiology.

A report, (Lindstrom et al., 1988b, later published as Luther et al., 1989) based on the electrophysiological analysis at the single channel level, indicated that AChRs on TE671 cells function like muscle AChRs and are blocked by α-BGT. Sine (1988) and Oswald et al. (1988) observed similar electrophysiological properties, confirming that the channel of TE671 cell AChR exhibited most of the general characteristics of electroplaque or skeletal muscle AChR channels, including those observed on cultured human

muscle cells.

Combined data from Sine (1988) and Luther et al (1989) based on the channel properties and reaction with monoclonal antibodies against extrajunctional AChRs suggest that AChRs from TE671 cells have extrajunctional type AChRs rather than junctional AChRs.

1.4.4.5. Protein chemistry; AChR subunit composition.

Luther et al. (1989) have affinity purified AChR from TE671 cells on an α-BGT affinity column and found that it is composed of four polypeptides corresponding to α, β, τ and δ subunits from Torpedo with apparent molecular weights of 42000, 52500, 55000 and 62000 Daltons. The acetylcholine binding sites were localised to the α-subunits, as shown by affinity labelling (see Section 1.2.4.4).

1.4.4.6. Molecular biology.

Work by Schoepfer et al., (1988) involved the cloning and sequencing of cDNA for the α-subunit of AChRs from TE671 cells. The sequence showed extensive homology to that of α-subunits of muscle AChRs from several species and is identical to the sequence predicted from a human genomic clone previously reported (Noda et al. 1983c). Schoepfer et al. (1988) also found that the acetylcholine binding subunit of AChRs from TE671 cells is distinct from that found on rat neuronal

AChRs and that sequence homology with PC12 cells and rat brain is lower than that for muscle. They concluded that the TE671 neuronal cell line expressed α-subunits identical to those of muscle rather than that of a neuronal type of AChR blocked by α-BGT.

1.4.4.7. AChR regulation.

Seigal and Lukas (1988) and Luther et al. (1989) noticed that regulation of TE671 cell AChR synthesis may differ from that of muscle cells. They observed that AChR expression is up-regulated by nicotinic agonists and sensitive to antagonists of α-BGT binding (Seigal and Lukas, 1988). This contrasts with muscle cells (Appel et al., 1981; 1982) in which the receptor is down regulated by prolonged agonist occupancy. Similar findings were reported by Robinson and McGee (1985) for AChRs of the neural-crest derived cell line PC12, although Whiting and Lindstrom (unpublished observations) note agonist up-regulation of the PC12 cell AChR. The mechanisms by which these agonists exert their effects are unclear, but regulation clearly varies in different cell types. It is possible that the TE671 cell AChR is under a "neuronal" type of regulation that may differ from that observed in muscle (Luther et al. (1989).

1.4.4.8. DNA fingerprinting.

TE671 cells express extrajunctional muscle-like AChRs in relatively large amounts (Luther et al., 1989). Why should "muscle-like" AChRs be expressed in a neuronal cell line?.

Stratton et al. (1989) very recently reported several lines of evidence suggesting that the TE671 cell line was misidentified when initially reported by McAllister et al. (1977). DNA fingerprinting techniques have revealed that TE671 is identical to the rhabdomyosarcoma cell line RD. Both cell lines were derived in the same laboratory but RD was described (McAllister et al., 1969) before the first report of TE671. These observations indicate that the TE671 cell line is most probably a subline of the rhabdomyosarcoma cell line RD, which would expain the presence of a "muscle-like" AChR on TE671 cells.

1.5. Outline and aims of the project.

Most of the evidence implicating antibody-dependent complement-mediated destruction of the muscle end plate in MG is either indirect or based on animal cells in culture (see, Table 1).

Previous investigations in this laboratory used the carnitine release assay described by Cambridge and Stern (1981), the results indicated that myasthenic sera

caused lysis of chick myotubes in culture (Bird, 1986) and rat myotubes in culture (Childs, 1985; Childs et al., 1984,1985,1987) whereas normal sera treated similarly did not.

In this project, using the same assay, the effects of myasthenic sera on fetal human skeletal muscle cells in culture were investigated. Human cultures proved to be an unreliable source of material for myotoxicity investigations so the studies were extended to include a human derived cell line (TE671) in culture. The results of this work are divided into two main sections: the first section describing studies on the cultured human muscle system and the second being concerned with TE671 cell cultures. The overall aim of the project was to develop a human model system to monitor antibody-dependent complement-mediated lysis in MG.

- MATERIALS.

Radiochemicals.

Carrier-free Na-[^{125}I] in dilute NaOH (100mCi/ml) was from Amersham International Plc, Amersham, Bucks, U.K. and was stored at room temperature for not longer than 3 weeks after its activity reference date.

L-[Me-^{3}H] carnitine hydrochloride (1mCi/ml, specific activity 87μCi/mmol) and Na_2-[$^{51}CrO_4$] in aqueous solution (1mCi/ml) were purchased from Amersham International Plc as above.

Counting instruments.

[^{125}I] was counted in an LKB 1280 Ultrogamma counter. Tritium and [^{51}Cr] were counted in a tritium-specific channel in a Packard Tri-carb scintillation counter (Model 3255) for a 2 minute counting period. Correction for quenching was made using the channels ratio method. The efficiency of counting for tritium was 38% and for [^{51}Cr] was 15%.

Ligands.

α-BGT from Bungaris multicinctus, decamethonium

bromide, carbamylcholine chloride, d-tubocurarine chloride, hexamethonium bromide, choline chloride, acetylcholine chloride and atropine sulphate were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

(-)-Nicotine hydrogen (+) tartrate was from BDH Chemicals, Poole, U.K.

Benzoquinonium chloride was a generous gift from Stirling Winthrop Inc., Renssalaer, New York, U.S.A.

Methyllycaconitine (MLA) citrate salt was a gift from Dr. M.H. Benn, Dept. Chemistry, University of Calgary, Alberta, Canada. (+)-Nicotine di-(-)-tartrate was provided by Dr. R. Barlow, Dept. Pharmacology, University of Bristol, Bristol, U.K.

Chemicals.

Standard laboratory reagents were from Sigma Chemical Co., Poole, Dorset, U.K. or from BDH Chemicals, Poole, Dorset U.K.

Gel filtration reagents were supplied by Pharmacia Ltd., Hounslow, U.K.

Ion-exchange resins, DEAE-cellulose filter discs, GF/C and GF/B glassfibre filter discs were from Whatman Lab. Sales Ltd., Maidstone, Kent, U.K.

Blue-gel was supplied by Amicon Ltd., Woking, Surrey, U.K.

Immunoreagents.

Serum samples from normal volunteers (Table 3) were obtained from colleagues in the Department and from Southmead Hospital, Bristol, U.K.. Myasthenic serum samples were obtained from patients (Table 4) in several hospitals in the U.K. as samples received for the routine assay of anti-AChR antibodies. Plasma resulting from plasmapheresis and plasma-EDTA samples were also obtained from Southmead Hospital, Bristol, U.K.. Non-EDTA plasma and serum samples were stored frozen at -20°C, EDTA-plasma samples were stored at -80°C.

Goat anti-(human-IgG) antiserum was prepared in the Department by repeated intramuscular injections of purified human IgG into a goat (Section 2.8.).

Rabbit anti-(fetal calf AChR) antiserum was a generous gift from Ms. S. Walsh of this Department.

Lyophilised guinea pig complement serum was obtained from ICN Immunobiologicals, Lisle, IL., U.S.A. (stated activity positive haemolysis at 0.07ml when the complement was at a 1/10 dilution), or from Flow Laboratories, Irvine, Ayrshire, Scotland (stated activity CH /ml =250, where one CH is the amount of complement producing 50% lysis of sensitised sheep red blood cells under stated conditions). The serum was reconstituted in the diluent provided and used immediately.

Anti-C3c complement was from DAKO-immunoglobulins,

Table 3. Details of normal controls used in this study.

Normal human serum	Age	Sex	Associated diseases
1	25	M	None
2	27	M	"
3	38	F	"
4	30	M	"
5	24	F	"
6	47	M	"
7	26	F	"
Pool	-	-	-

Table 4. Details of myasthenic patients used in this study.

Patient	Age	Sex	Severity of symptoms	Thymectomy	Therapy	Anti-AChR antibody titre (nM)
1	-	F	G	-	-	42.00
2	-	F	-	-	-	a)20.60 b)17.5
3	32	F	S/G	T_x	IS	a)71.3 b)79.3
4	38	F	S/G	T_x	IS,P_x	10.90
5	21	M	O	T_x	-	0.26
6	-	M	-	-	-	9.00
7	64	M	-	-	-	76.10
8	21	F	-	-	-	9.60
9	62	F	Crisis	Thymoma	-	8.58
10	66	F	-	-	-	5.41
11	-	F	-	-	-	0.22
12	68	M	-	-	IS,P_x	24.60
13	21	F	-	-	IS	35.10
14	-	M	-	-	-	54.00
15	51	F	-	-	-	5.28
16	52	M	-	-	-	1.04
17	19	F	-	-	-	8.59
18	61	M	-	-	-	6.26

G = Generalised myasthenia
S/G = Severe generalised myasthenia
O = Ocular myasthenia
Crisis = Myasthenic crisis
IS = Immunosuppressive therapy
P_x = Plasma exchange
T_x = Thymectomy

a)Frozen serum
b)Fresh serum

Denmark and rabbit anti-human C3d was from Southmead Hospital, Bristol, U.K.

Source of muscle AChR.

Human adult muscle was supplied by the Royal United Hospital, Bath U.K. and Bristol Royal Infirmary, Bristol, U.K., following lower limb amputations resulting from severe vascular disorders. Within 15 min of the operation, calf muscle was crudely dissected free from fat, tendon and skin, transported in ice, solid carbon dioxide or liquid nitrogen and stored at -80°C for up to six months.

Source of muscle and cells for tissue culture.

Human fetal tissue was obtained from fetuses (approximate age 8-16 weeks) from the Royal United Hospital, Bath, U.K., following pregnancy terminations by suction on a vacuum line. As soon as possible after the operation, the fetal limbs were placed in growth medium and stored at 4°C for up to 72h.

TE671 cells were kindly provided by Prof. J. Newsom-Davis, John Radcliffe Hospital, Oxford, U.K.

Tissue culture reagents.

Dulbecco's modified Eagle's medium (DMEM) and 2.5% (w/v) crude trypsin solution, in Hank's balanced salt solution, were obtained from Flow Laboratories, Irvine, Ayrshire, U.K.

Donor horse serum (DHS) and fetal calf serum (FCS) were obtained from Gibco Ltd., Uxbridge, Middlesex, U.K. and were heat inactivated (56°C, 30min) before use. Penicillin (5000U/ml) and streptomycin (5000µg/ml) solution, L-glutamine (200mM), kanamycin (10µg/ml), fungizone (amphotericin B, 250µg/ml) antibiotic-antimycotic solution (100X, 10000U/ml penicillin, 10000U/ml streptomycin and 25µg/ml fungizone) and trypsin-EDTA 10X, were obtained from Gibco Ltd., as above.

Deoxyribonuclease, hormones and co-factors were supplied by Sigma Chemical Co., Poole, Dorset, U.K. Glucose and sucrose (analytical grade) were obtained from BDH Chemicals Ltd., Poole, U.K. All reagents supplied in a non-sterile form were sterilised before use by passage of stock solutions through sterile filters (0.2µm) from Sera Lab., Crawley Down, W. Sussex, U.K. All sterile plastic ware used for tissue culture was supplied by Nunc Gibco Ltd., Uxbridge, Middlesex, U.K.

Nylon bolting cloth (0.53µm aperture) was from Henry Simon Ltd., Stockport, Cheshire, U.K.

All tissue culture procedures were carried out in an

Intermed Pathfinder laminar flow cabinet.

Media.

The compositions of the tissue culture growth media and balanced salt solutions (BSS) used, were as follows:

Human muscle cell culture medium.

DMEM supplemented with:-

Fetal calf serum	20.0% (v/v)
Glucose	0.15% (w/v)
L-Glutamine	2.00mM
Penicillin	100U/ml
Streptomycin	100µg/ml

TE671 cell medium.

DMEM supplemented with:-

Fetal calf serum	10.0% (v/v)
L- glutamine	2.00mM
Penicillin	100U/ml
Streptomycin	100µg/ml
Kanamycin	0.02µg/ml

Growth media were stored at 4°C and used within 4 weeks.

Balanced salt solutions.

Puck's BSS.

NaCl	8.0g
KCl	0.4g
Na_2HPO_4	0.024g
Glucose	6.0g
Sucrose	15.0g

The above were made up to 1L with double distilled water containing 0.01M HEPES, final pH 7.3.

Phosphate buffered saline (PBS).

NaCl	8.0g
KCl	0.2g
Na_2HPO_4	0.2g
KH_2PO_4	1.1g

The above were made up to 1L with double distilled water.

Hank's balanced salt solution (HBSS).

NaCl	8.0g
KCl	0.4g
$MgSO_4.7H_2O$	0.1g
Na_2HPO_4 anhyd.	0.048g
$CaCl_2.2H_2O$	0.185g
$NaHCO_3$	0.35g
KH_2PO_4	0.06g
$MgCl_2.6H_2O$	0.1g

The above were made up to 1L with double distilled

water.

METHODS.

2.0. General biochemistry.

2.1. Iodination of α-BGT.

α-BGT was labelled with [^{125}I] by the chloramine-T method (Hunter, 1967) as modified by Urbaniak et al. (1973). Carrier-free Na-[^{125}I] (100mCi/ml) in dilute sodium chloride (20µl) was added to α-BGT (20µg,2.5nmol) in potassium phosphate buffer, pH7.5, (20µl), followed by chloramine-T (0.5% w/v) in 0.05M potassium phosphate buffer pH7.5 (10µl). The mixture was stirred for 1 min. at 23°C, after which the reaction was terminated by the addition of 0.016% (w/v) sodium metabisuphate in 0.05M potassium phosphate buffer, pH7.5 (750µl), followed by 1% (w/v) carrier potassium iodide in the same buffer (200µl).

The labelled protein was separated from the unreacted [^{125}I] by passage through a column of Sephadex G-25 (25 x 1cm) previously equilibrated in 0.01M potassium phosphate buffer pH7.5, containing 1% (w/v) BSA. Fractions (1ml) were collected and aliquots (5µl) from each fraction were counted for radioactivity. The peak fractions containing [^{125}I] α-BGT were pooled and stored at 4°C for not longer than three weeks. The specific radioactivity of the [^{125}I] α-BGT was calculated assuming 100% recovery of the protein.

A typical elution profile from the G-25 column is

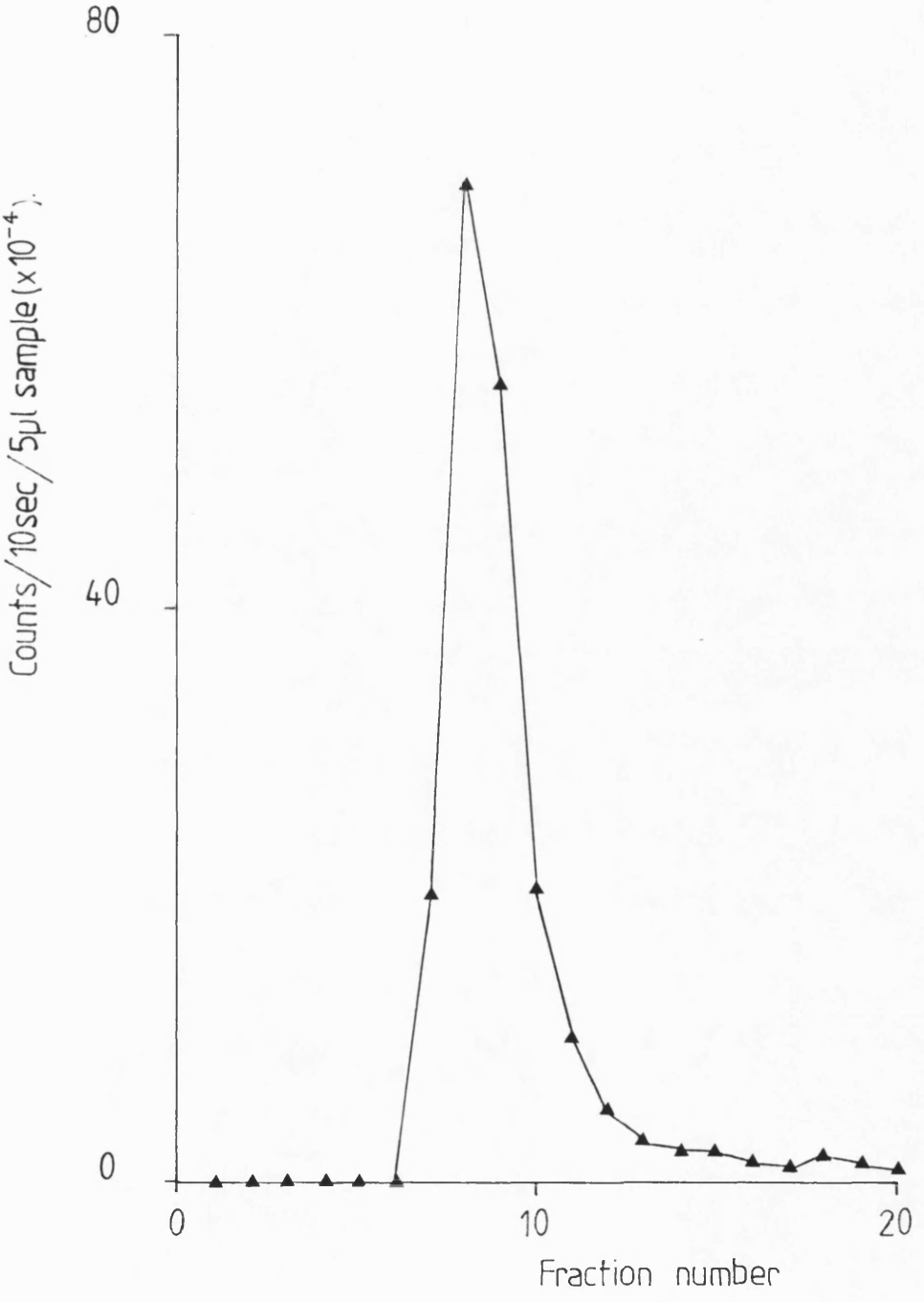
shown (Fig. 9), where it can be seen that approximately 90% of the [^{125}I] was incorporated into the protein [range 75-98%; mean ± SEM (n): 90 ± 2.44% (10)]. The specific activity was in the range 594-899 Ci/mmol [mean ± SEM (n): 732 ±26 (10) Ci/mmol].

2.2. Biological activity of [^{125}I] α-BGT.

The biological activity of the [^{125}I] α-BGT preparations was determined by measurement of the proportion of radiolabelled α-BGT that could be bound by a large molar excess of purified AChR from Torpedo marmorata.

Triplicate samples of Torpedo AChR (30pmol, 100µl) in 0.01M potassium phosphate buffer, pH7.4, containing 0.1% (w/v) BSA, 1% (v/v) Triton X-100 and 0.01% (w/v) sodium azide, were incubated with the test radiolabelled α-BGT (0.5pmol, 50µl) for 90 min at 23°C in the presence and absence of excess unlabelled α-BGT (3µM).
Bound [^{125}I] α-BGT was separated from free toxin on DEAE cellulose filter discs essentially as described by Schmidt and Raftery (1973). Each sample was applied to 2 DEAE-81 cellulose filter discs (diameter 24mm) premoistened with the above assay buffer and left to stand for 2 min. Samples were then vacuum filtered on a Millipore filter unit. The filters were washed (3 x 1ml) with the assay buffer and counted for radioactivity. Biological activity was calculated as follows:-

FIG. 9 Gel filtration of [^{125}I]α-BGT on Sephadex G-25.



$$\text{Biological activity (\%)} = \frac{\text{specific cpm bound (sample)}}{\text{total cpm added}} \times 100$$

The biological activity of the [^{125}I] α-BGT preparations was in the range 32-60% [mean ± SEM (n): 47 ± 2.44% (10)].

2.3. Preparation of a crude extract of human AChR.

Detergent extracts of muscle were prepared from human adult legs according to the method of Stephenson et al. (1981).

Muscle (250g), was coarsely chopped and homogenised in a Waring blender at maximum speed (2 x 1 min.) in 0.01M potassium phosphate buffer, pH7.4, (4 vols.) containing 1mM EDTA, 2mM benzamidine hydrochloride, 0.1M benzethonium chloride, 0.1mM PMSF, bacitracin and 0.02% (w/v) sodium azide. The homogenate was centrifuged (10000 g, 45 min, 4°C) and the resulting supernatant was decanted and discarded. The pellet was resuspended in the same buffer (1 vol.) containing additionally 2% (v/v) Triton X-100 (extraction buffer), stirred for 4h at 23°C and centrifuged (100000 g, 60 min., 4°C). The resulting supernatant was filtered through glass wool. This crude muscle extract was used in the radioimmunoassay (Section 2.6.) for anti-AChR antibodies in human serum.

2.4. Determination of AChR content of muscle extracts.

2.4.1. Ammonium sulphate precipitation.

The concentration of AChR from solubilised human muscle was determined by a method adapted from that of Meunier et al. (1972). Triplicate samples of human muscle extract (100µl) were incubated with 2.5nM [^{125}I] α-BGT in extraction buffer (50µl) for 45 min at 23°C. Specific binding of [^{125}I] α-BGT was blocked in parallel incubations containing 1mM benzoquinonium chloride. Saturated ammonium sulphate was added to give a final concentration of 40% (w/v). After further incubation for 16h at 4°C, the precipitates were collected on Whatman GF/C glassfibre filter discs, washed with 40% (w/v) saturated ammonium sulphate solution (3 x 1ml) by vacuum filtration on a Millipore filter unit and counted for radioactivity.

Detergent extracts of human skeletal muscle, measured by the ammonium sulphate method, gave yields of 0.26-3.9pmols [^{125}I] α-BGT binding sites per gram of muscle (mean ± SEM (n): 1.41 ± 0.48 (6)).

Determination of AChR, in the extract, by using precipitation with ammonium sulphate, gave high non-specific binding. Accordingly an alternative method, adapted from Bruns et al. (1983) was investigated.

2.4.2. Polyethylenimine treated filter method.

Triplicate samples of human muscle extract were

incubated as described above.

Non-bound toxin was separated from toxin-receptor complexes on polyethylenimine (PEI) pretreated Whatman GF/B glassfibre filters using a method adapted from Bruns et al. (1983).

A stock solution of PEI was made by diluting commercial 50% PEI free base 1:5 in PBS. Filters were soaked in fresh 0.3% PEI (1:33 dilution of stock 10% PEI) in PBS for 1-24h before use. Samples were applied to the filters and washed with PBS (3 x 1ml) by vacuum filtration on a Millipore filter unit and counted for radioactivity.

Results obtained by using this method yielded much lower levels (10-20%) of non-specific binding than did the ammonium sulphate precipitation assay. The polyethylenimine assay is now routinely used in assaying AChR from purified muscle preparations.

In both methods (2.4.1. and 2.4.2.) sufficient excess of [^{125}] α-BGT was ensured by repetition of the assay using serial two-fold dilutions of receptor in extraction buffer, when a linear relationship between dilution number and radioactivity should be obtained. The AChR content of the crude muscle extract was calculated in terms of [^{125}I] α-BGT binding sites as follows:-

$$\text{AChR (pmol/ml)} = \frac{\text{specific cpm}}{\text{cpm/pmol [}^{125}\text{I] α-BGT}} \times \text{sample dilution} \times 10$$

2.5. Preparation of rat brain P2 membrane fraction.

Brain tissue homogenate was prepared essentially as described by Gray and Whittaker (1962) with modifications by MacAllan et al. (1988).

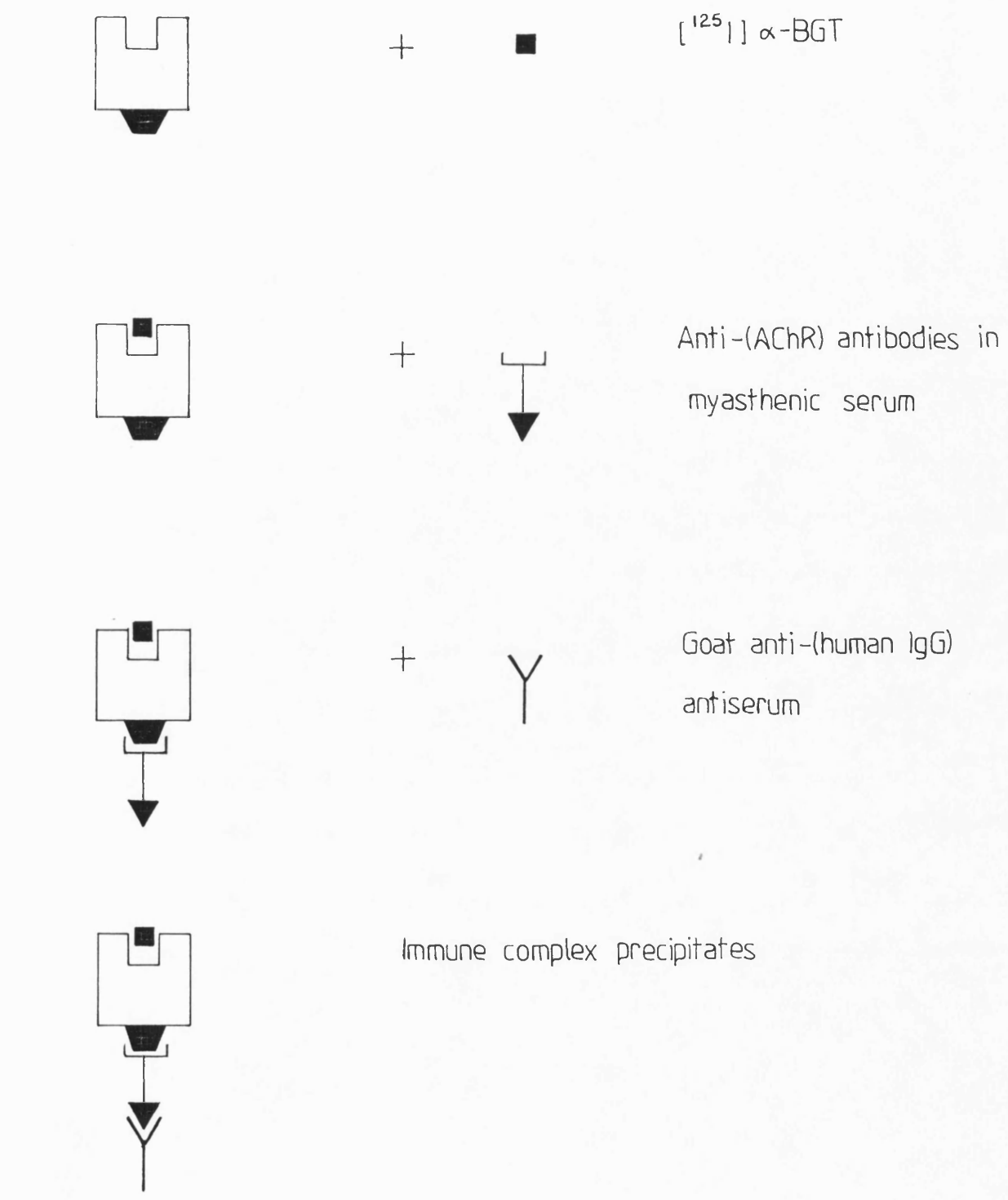
Whole brains of adult Wistar rats (minus the cerebellum) were homogenised (10% w/v) in 0.32M sucrose solution, pH7.4, containing 1mM EDTA, 0.1mM PMSF and 0.01% (w/v) sodium azide and the suspension was centrifuged at 1000 g for 10 min. The supernatant was decanted and retained on ice. The pellet was resuspended in 0.32M sucrose (5ml/g original wet weight) and recentrifuged. The supernatants were combined and centrifuged at 12000 g for 30 min to give a P2 pellet. This was resuspended in 50mM potassium phosphate buffer, pH7.4, containing protease inhibitors as above, to give a final volume of 2.5ml/g original wet weight and washed twice by centrifugation and resuspension.

2.6. Radioimmunoassay of anti-(AChR) antibodies.

A radioimmunoassay procedure similar to that previously described (Lindstrom et al., 1976: Carter et al., 1981) was used to determine the anti-AChR antibody content of fractionated serum samples. This procedure detects anti-AChR antibodies not directed at the α-BGT binding site.

Detergent extract of human leg muscle (0.05pmol specific α-BGT binding sites) was labelled by incubation with excess [^{125}I] α-BGT (0.5pmol) for 45 min at 23°C. Specific binding of [^{125}I] α-BGT was blocked in parallel incubations with 1mM benzoquinonium chloride. The resulting solutions were incubated in triplicate with serum or serum fractions (5µl) appropriately diluted with normal human serum, for 2h at 23°C (or 16h at 4°C). The labelled AChR-Ab complex was precipitated by the addition of goat anti-(human IgG) antiserum (35µl, the amount chosen to ensure maximum precipitation of the complex) and incubation for 2h at 23°C (or 16h at 4°C) (see Fig. 10). The resulting precipitates were collected by centrifugation (3000 g, 10 min, 4°C) and the pellets washed twice with 10mM potassium phosphate buffer, pH7.4, containing 0.15M sodium chloride, 1% (w/v) Triton X-100 and 0.1% (w/v) sodium azide (RIA buffer) by alternate suspension and centrifugation, then counted for radioactivity. For each sample, maximum formation of [^{125}I] α-BGT antibody complexes was ensured by repetition of the assay using serial two-fold dilutions of the sample in order to obtain a linear relationship between the volume of undiluted sample and precipitated radioactivity. The anti-AChR antibody titre of serum or serum fractions is expressed as moles of specific [^{125}I] α-BGT binding sites precipitated per litre of sample as follows:-

FIG. 10 Diagrammatic representation of the radioimmunoassay for anti-(AChR) antibodies.



$$\text{Titre (AChR)} = \frac{\text{specific cpm sample}}{\text{specific radioactivity}} \times \text{dilution factor}$$

cpm/pmol [^{125}I] α-BGT

2.7. Purification of IgG.

IgG was prepared from normal and myasthenic serum by the method of Stevenson and Dorrington (1970). A solution of saturated ammonium sulphate (6ml saturated in 0.2M Tris-HCl, pH8.0) was added dropwise to normal or myasthenic serum (10ml) with stirring at 23°C. The solution was stirred for a further 30 min at 23°C and the precipitate was sedimented by centrifugation (500 g, 15 min). The precipitate was dissolved in 30mM potassium phosphate buffer, pH7.3 (10ml) and dialysed overnight against the same buffer (4L). The non-dialysable material was applied to a column (20 x 2.5cm) of DE-52 cellulose pre-equilibrated with 30mM potassium phosphate buffer, pH7.3. The column was eluted with the same buffer and fractions (2ml) collected. Fractions showing absorbance at 280nm were pooled and concentrated on an Amicon B15 concentrator to the original volume of serum (10ml). The column was washed with 2M sodium chloride and re-equilibrated with 30mM potassium phosphate buffer before re-use. Purified IgG was stored at -20°C until required.

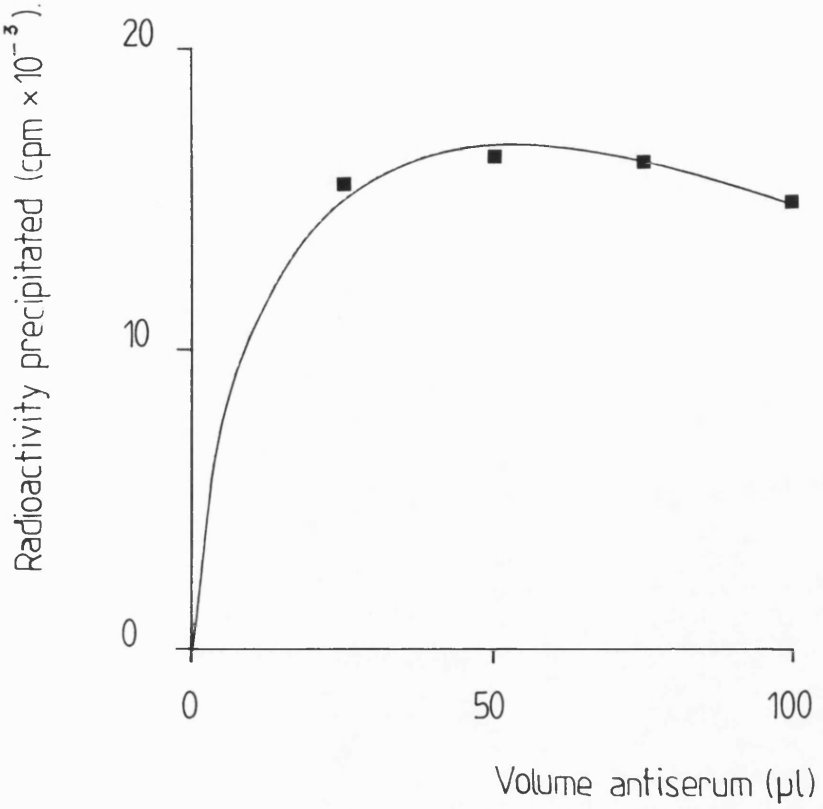
2.8. Preparation of goat anti-(human IgG) antiserum.

Heat aggregated (60 min, 56°C) normal human IgG (Hagg), (1.0ml, 1.5mg/ml protein) was emulsified with Freund's complete adjuvant (1.0ml) and injected intramuscularly at one site in each hind leg of a goat. Repeated injections of Hagg in Freund's incomplete adjuvant were performed at 3 weekly intervals. A blood sample (10ml) was taken from the goat before each injection to allow the determination of goat anti-(human IgG) antibodies. When an adequate titre was reached, the goat was bled out. The blood was allowed to clot, at 4°C, overnight then centrifuged (5000 g, 20 min, 4°C). The serum was collected and stored at -20°C in the presence of sodium azide.

An adequate titre of goat anti-(human IgG) antibody was where less than 100µl of antiserum was needed to effect total precipitation of [^{125}I] α-BGT labelled AChR-antibody complex obtained by incubation of a fixed volume (5µl) of myasthenic serum, with a constant amount of [^{125}I] α-BGT labelled adult human AChR in the presence and absence of benzoquinonium chloride (for details, see Section 2.6.).

A typical saturation curve is shown in Fig.11. Injections were repeated until an adequate titre was attained. In each of two goats, 3 injections were sufficient to achieve titres such that 35µl of antiserum effected precipitation of labelled AChR-antibody complex as outlined above. 600ml of antiserum was obtained from

FIG. 11 Saturation curve for the precipitation of receptor-antibody complexes by increasing volumes of anti-(human IgG) antiserum.



each goat.

2.9. Estimation of C3 degradation products.

Barbitone buffer.

Solution A contained sodium barbitone (20.6g), EDTA (3.72g) in distilled water (500ml). Solution B contained barbitone (3.68g) boiled to dissolve in distilled water (250ml).

Solutions A and B were mixed, allowed to cool and the pH adjusted to 8.65 with 5M NaOH, before dilution to 1 litre.

Buffer for electrophoresis chamber:		dilute stock 1/2
Buffer for gel:		dilute stock 1/4
Destain solution:	water	450ml
	methanol	450ml
	glacial acetic acid	100ml
Protein stain:	Brilliant Blue R (5g) in above solvent, filtered prior to use.	

Estimation of C3d in serum samples was measured by double decker rocket immunoelectrophoresis, similar to that described by Brandsland et al. (1981), as modified by Bedwell et al. (1986).

Agarose 1% (w/v) in barbitone buffer pH8.5 (see above) and that containing additionally either anti C3c antiserum (DAKO immunobiologicals), (40µl/ml gel) or polyclonal anti-(human C3d), prepared at Southmead Hospital, Bristol, (50µl/ml gel) were prepared and stored in a water bath, at 56°C, immediately prior to

use. The gels were poured onto Gel Bond film (Fig. 12) and allowed to set.

Myasthenic serum samples collected in EDTA (3µl) and five dilutions of a standard serum (3µl, prepared from normal human serum incubated at 37°C for 4h to allow the complete conversion of C3 to C3c and C3d) were applied to the gel. Samples were electrophoresed in an LKB Multiphor electrophoresis tank at 2.5V/cm overnight at 4°C. After this time, the gel was removed, washed extensively in 0.14M saline and pressed. The gel was completely dried with a hair dryer before staining in Brilliant Blue R (2 min., 23°C) and destaining to give a colourless background.

Results are expressed as a percentage of the amount of C3d in the standard serum.

2.10. TISSUE CULTURE.

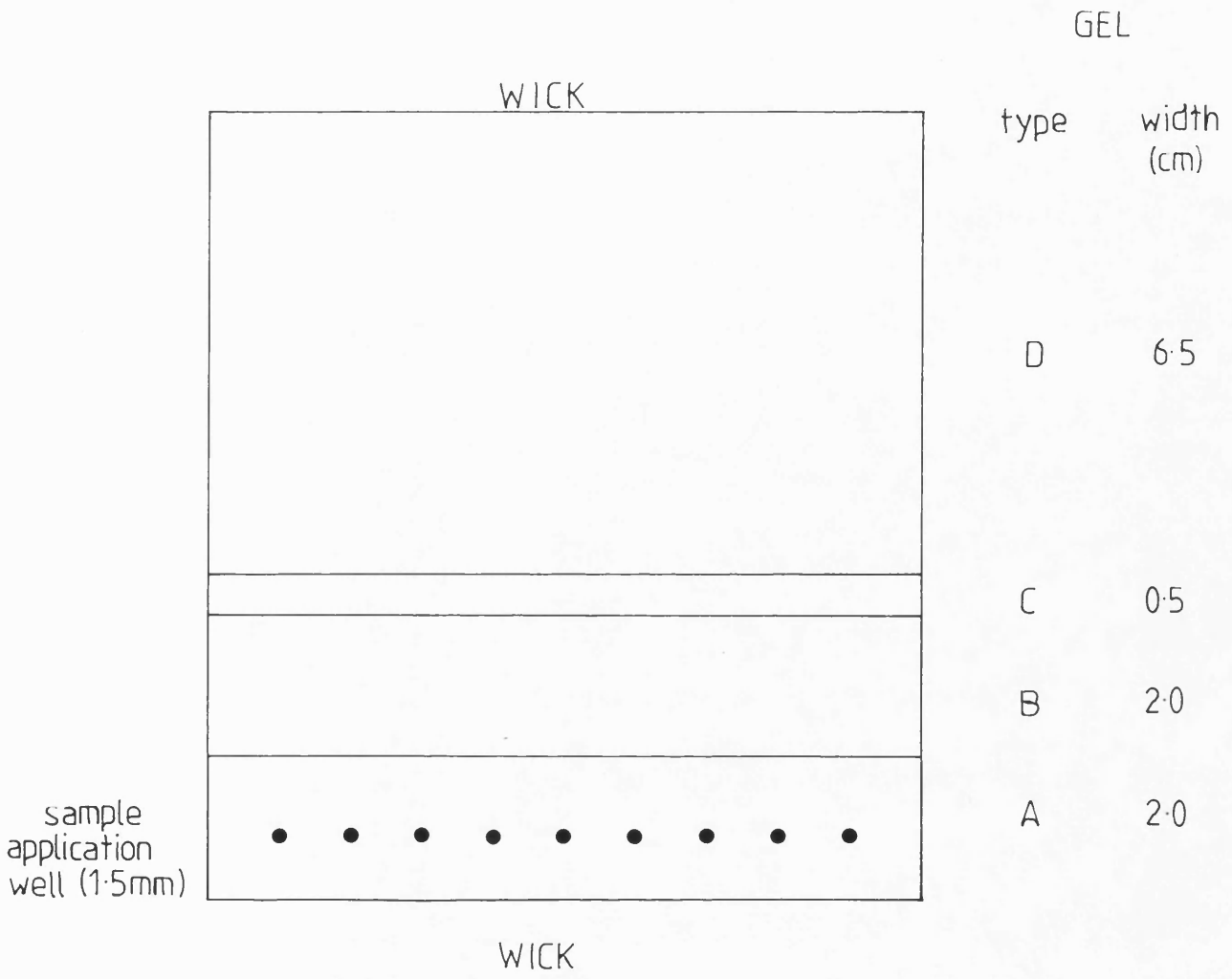
2.10.1. Human myotube cultures.

2.10.2. Preparation of tissue culture plates.

Collagen was prepared essentially according to Erhman and Gey (1956).

A rat's tail was washed in 70% ethanol for 30 min. The skin was removed and the tendons dissected out with bone forceps. The tendons were washed in distilled water and placed in 0.1% (v/v) glacial acetic acid (100ml) at 4°C for 48h to extract the collagen. The resulting solution was centrifuged at 800 g for 2h at

FIG. 12 Organisation of gel bond for immunoselection.



Gels A and C native agarose

Gel B contains anti-C3c antibody

Gel D contains anti-C3d antibody

23°C. The resulting supernatant was diluted with distilled water to give a protein concentration of 0.75mg/ml and stored at 4°C until required.

Tissue culture plates (24 well) were precoated with photo-reconstituted collagen gel according to the method of Masurovsky and Peterson (1973).

Collagen solution (0.75mg/ml) was mixed with riboflavin 0.05% w/v) in a ratio of 4:1 (v/v). A sample of this solution (30µl) was spread over the surface of each culture well. The gel was photopolymerised by exposure to fluorescent light for 1h in a laminar flow cabinet. Coated plates were dried overnight at 37°C. The plates were washed with sterile distilled water (1-1.5ml) for 2-3 min and incubated with growth medium for at least 30 min before the addition of cells.

2.10.3. Establishment of cultures.

Human myotube cultures were established using muscle tissue from the limbs of 8-16 week fetuses essentially according to the method of Yaffe (1973) for neonatal rat muscle cultures.

Limbs were washed in Ca^{++} and Mg^{++} free balanced salt solution (Puck's BSS, see "Materials"). Dissection of limbs was then carried out under a Swift binocular viewer at x10 magnification. After removal of skin, tissue surrounding the bone was stripped and placed in a 35mm plastic petri dish containing Puck's BSS, (0.5ml). The tissue was minced with a pair of irridectomy scissors to produce a slurry and transferred with

washing to a sterile plastic tube (final volume 8.5ml). Deoxyribonuclease (1mg/ml, 0.5ml) was added to prevent cell clumping, induced by deoxyribonucleic acids released from dead cells. Crude trypsin solution (2.5% w/v, 1ml) was then added to give a final concentration of 0.25% (w/v). The tissue suspension was incubated at 37°C for 60 min. with periodic mixing. The suspension was then centrifuged at 400 g for 5 min, the supernatant was discarded and growth medium (5ml, see "Materials") was added to the resulting pellet. Any residual trypsin action was inhibited by the serum component of the growth medium. Cells were then released from the tissue fragments by gentle trituration with a glass pasteur pipette (15-20 cycles). The suspension was left to stand for 3 min and the supernatant, containing free cells, was removed for filtering. Further growth medium was added to the remaining tissue and the trituration process was repeated.

The combined cell suspensions were filtered through two layers of nylon bolting cloth (53µm aperture) to remove remaining cell aggregates and tissue clumps. A sample of the filtrate was added to an equal volume of trypan blue (0.2% w/v in PBS) before counting in a haemocytometer.

Cells were added to collagen coated 24 well (15.5mm) culture plates at a final plating density of 2.5×10^5 cells/well (1.3×10^6 cells/cm^2) in growth medium (1ml).

Cultures were grown in a humidified atmosphere of 10% CO_2/air. Growth medium was replaced by fresh, pre-

warmed medium every three days. After 3-4 days growth, when fusion of myoblasts was judged morphologically to be complete, cytosine arabinoside (10μM) was added to the cultures for 72h. This mitotic inhibitor reduced the growth of fibroblasts in the cultures.

2.10.4. Carnitine assay: mytoxicity of serum and complement.

L-[Me-^{3}H]-carnitine hydrochloride of high specific activity (87μCi/mmol, radioactive concentration, 1mCi/ml) was diluted, under sterile conditions, in growth medium to a final concentration of 0.23μM.

Quadruplicate myotube cultures in 24-well plates, prepared, as described above, were labelled by incubation with L-[Me-^{3}H]-carnitine for 18h at 37°C in an atmosphere of 10% CO_2/air, after which the cultures were washed three times with growth medium (0.5ml, 2-3 min). After washing, fresh medium was added to each culture well followed by the addition of heat inactivated test serum (0.08ml) and finally lyophilised guinea pig serum (0.08ml). The cultures were then incubated at 37°C in an atmosphere of 10% CO_2/air for 3h. After the incubation, cultures were washed with growth medium (3 x 0.5ml, 2-3 min), solubilised with 0.1M sodium hydroxide, mixed with scintillation fluid (Optiphase Safe, 5ml) and counted for radioactivity.

Control cultures to which no additions of test serum or guinea pig serum were also included in the test.

Myotoxicity was expressed as the percentage loss of radioactivity, compared with controls, according to the formula:-

$$\frac{CRC - CRT}{CRC} \times 100\%$$

Where CRC = counts retained in control cultures to which no additions of serum were made.

CRT = counts retained in test cultures.

2.11. TE671 cell culture.

The TE671 cells were routinely cultured in growth medium (see "Materials") in 80cm^3 flasks at 37°C in an atmosphere of 10% CO_2/air.

2.11.1. Preparation of tissue culture plates.

Tissue culture plates (24 well and 96 well) were pre-coated with polylysine solution (5µg/ml, 1ml) overnight at 37°C. The polylysine solution was removed by aspiration and each well washed with sterile distilled water (1-1.5ml, 24 well plates; 100-150µl, 96 well plates) for 2-3 min and incubated with growth medium for at least 30 min before the addition of cells.

2.11.2. Cell harvesting.

TE671 cells, in 80cm^3 flasks, were harvested with trypsin-EDTA.

The medium above the cells was removed by

aspiration and the cell layer washed gently with warmed, sterile PBS (10ml). The PBS was removed by aspiration and trypsin-EDTA (0.25% w/v trypsin, 2% w/v EDTA in PBS) was added to each flask for 30 sec, with agitation. The trypsin-EDTA was then removed by aspiration and the flasks were incubated for 5 min. at 37°C. TE671 cells were collected in PBS (2 x 10ml) and a sample of the resulting suspension was added to an equal volume of trypan blue (0.2% v/v in PBS) before determining viable cell numbers in a haemocytometer. The cells were then centrifuged (500 g, 5 min), resuspended in fresh, warmed growth medium and seeded out into prepared tissue culture plates (as above) at cell densities of 5 x 10^5 cells/ml (24 well plates) or 5-10 x 10^3 cells/100µl (96 well plates). Any remaining cells were returned to 80cm^3 flasks for continuous culture, or stored frozen at - 80°C, in DMEM containing 50% (v/v) FCS and 20% (v/v) DMSO.

Protein was determined according to the method of Lowry et al. (1951), after washing the cells with PBS to remove growth medium and solubilisation of the cells in 0.1M sodium hydroxide.

2.11.3.1. Characterisation of TE671 cell AChR.

2.11.3.2. Determination of AChR content of intact and solubilised TE671 cell extract.

The concentration of AChR in intact and solubilised TE671 cells was determined by using a method adapted

from Bruns et al. (1983).

Cells were harvested as described above and triplicate samples (100µl) of intact cells in PBS or cell extract in PBS containing additionally 1% (w/v) Triton X-100, were incubated with 2.0nM [^{125}I] α-BGT in PBS (+/- Triton X-100) for 90 min at 23°C. Specific binding of [^{125}I] α-BGT was blocked in parallel incubations using d-tubocurarine (1.0mM) or α-BGT (1µM). Non-bound toxin was separated from toxin receptor complexes on PEI pretreated Whatman GF/B glassfibre filters and the AChR content of the crude cellular extract was calculated in terms of [^{125}I] α-BGT binding sites as previously described (Section 2.4.).

2.11.3.1. [^{125}I] α-BGT binding to TE671 cells.

[^{125}I] α-BGT binding was measured in intact cells. Cells were harvested, as previously described. Cells (5 x 10^5) were replated in growth medium (1ml) in each well of a 24 well plate (15.5mm diameter). Binding was assayed the following day when the cells were firmly attached and judged morphologically to be confluent. [^{125}I] α-BGT in growth medium (0.5-10nM , final concentration) was added to the cells and incubated for 2h at 37°C. Non-bound toxin was removed by aspiration of the medium, followed by washes with growth medium (3 x 0.5ml). Non-specific binding was determined by pre-incubating (30 min) the cells with d-tubocurarine chloride (1mM, final concentration). The cells were

solubilised with 0.1M sodium hydroxide and counted for radioactivity in an LKB 1280 Ultrogamma counter. Specific [^{125}I] α-BGT binding to the cultures was calculated from:-

$$\frac{\text{total cpm (-d-TC)} - \text{non-specific cpm (+d-TC)}}{\text{cpm/pmol [}^{125}\text{I] α-BGT}}$$

2.11.3.4. Determination of apparent constants for inhibition by cholinergic ligands, of binding of [^{125}I] α-BGT to TE671 cells.

Cells were harvested and replated in fresh growth medium as previously described.

Triplicate wells were incubated with [^{125}I] α-BGT (20µl, 2.0nM final concentration) in the presence of increasing concentrations of unlabelled cholinergic agents (100µl) for 90min, 37°C, with final concentrations as follows:-

α-bungarotoxin	$1x10^{-10}$ -- $2.5x10^{-8}$M
decamethonium	$1x10^{-8}$ -- $1x10^{-5}$M
d-tubocurarine	$1x10^{-8}$ -- $2.5x10^{-6}$M
benzoquinonium	$2.5x10^{-7}$ -- $1x10^{-4}$M
methyllycaconitine	$1x10^{-6}$ -- $2.5x10^{-4}$M
acetylcholine	$1x10^{-7}$ -- $2.5x10^{-5}$M
carbachol	$2.5x10^{-8}$ -- $1x10^{-5}$M
(-) nicotine	$1x10^{-7}$ -- $2.5x10^{-4}$M
(+) nicotine	$2.5x10^{-7}$ -- $1x10^{-4}$M

glutamate, glycine, atropine, choline and hexamethonium

were all tested at a concentration of $1x10^{-3}M$.

After the incubation period, the medium above the cells was removed by aspiration, the cells were washed with growth medium (3 x 0.5ml) and extracted with 0.1M NaOH (2 x 0.5ml). The combined extracts were counted for radioactivity in an LKB 1280 Ultrogamma counter. The amount of binding was calculated, for each concentration point, and plotted, as a percentage of control binding, [determined in a parallel incubation using a molar excess of unlabelled α-BGT (1μM)], against log of displacing drug concentration.

2.11.3.5. Determination of apparent constants for inhibition by MLA, of [^{125}I] α-BGT binding to various tissue preparations.

a) Frog and human muscle extracts.

Detergent extracts of frog muscle (Rana pipiens) with a protein concentration of 15.5mg/ml were kindly provided by Dr. S. Wonnacott. Detergent extract of human muscle was prepared, as previously described (Section 2.3.).

Triplicate samples of extract (500μl, 1.5mg/ml protein) were incubated with [^{125}I] α-BGT (20μl, 2.0nM) in the presence of increasing concentrations of MLA (20μl, $2.5x10^{-7}$ -- $1x10^{-3}M$), 90 min, 23°C. After the incubation, non-bound toxin was separated from toxin receptor complexes on PEI pretreated Whatman GF/B glassfibre filters and counted for radioactivity (for details, see Section 2.4.2.).

b) Rat brain P2 membranes.

Rat brain P2 membranes were prepared as previously described (Section 2.5.). Binding of [^{125}I] α-BGT to these membranes was assayed essentially as described by Schmidt (1977).

The membrane fraction was diluted 10-fold in 50mM potassium phosphate buffer, pH7.4, and triplicate samples (0.5ml) were incubated with [^{125}I] α-BGT (20µl, 2.0nM) in the presence of increasing concentrations of MLA (20µl, $2.5x10^{-11}$ -- $1x10^{-8}$M), for 3h at 23°C. After the incubation, non-bound toxin was separated from toxin receptor complexes by centrifugation (2 min, 10000 g) in an MSE Microcentaur bench centrifuge. The pellet was washed once with ice-cold PBS and counted for radioactivity as above.

The amount of specific binding for each concentration point was calculated and plotted as a percentage of control binding determined in parallel incubations using a molar excess of unlabelled α-BGT (10µM) against log displacing drug concentration.

2.11.3.5. Inhibition of [^{125}I] α-BGT binding to TE671 cells by myasthenic serum.

Cultures were set up as for [^{125}I] α-BGT binding (Section 2.11.3.3.). An additional pre-incubation step

with myasthenic serum (the dose range of which was dependent on the anti-AChR antibody titre) was introduced after the incubation with d-tubocurarine chloride. Control wells to which no serum or normal human serum was added, were included in each test. The cells were washed, solubilised and counted for radioactivity as previously described.

Inhibition of [^{125}I] α-BGT was determined as follows:-

$$\frac{\text{specific cpm test serum}}{\text{specific cpm control serum}} \times 100\%$$

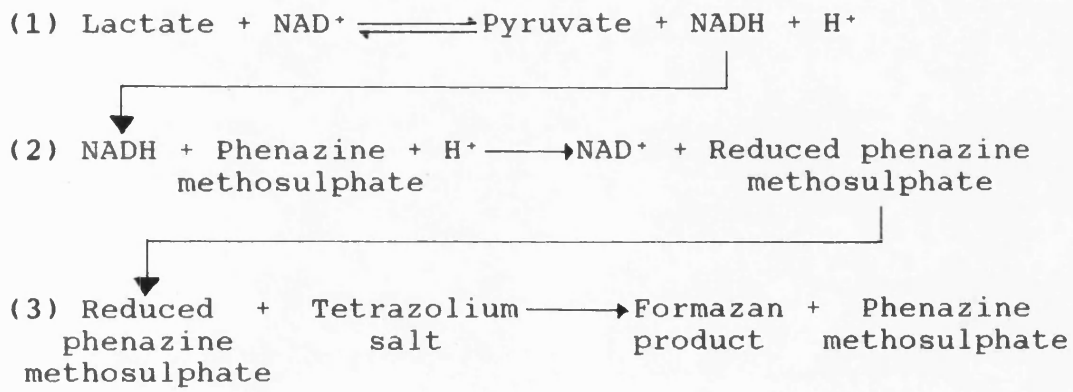
2.11.4. TE671 cell cytotoxicity studies.

2.11.4.1. Lactate dehydrogenase [EC 1.1.1.27] assay.

The presence of LDH [EC 1.1.1.27] in TE671 cells was detected using a colorimetric assay based on that described by Bergmeyer and Bernt (1974) (Fig.13).

Cells were harvested as previously described (Section 2.11.2.) but resuspended in Hank's BSS (HBSS), containing additionally Triton X-100 (2% v/v). This suspension was serially diluted in HBSS and quadruplicate samples (100µl) were plated into each well of a 96 well plate (6.0mm diameter). Lactate solution, containing lactate, 5.4×10^{-2}M; p-iodonotrotetrazolium violet, 6.6×10^{-4}M and phenazine methosulphate, 2.5×10^{-4}M in distilled water (25ml) was diluted (five-fold) in

Fig. 13. Principle of the colorimetric assay for LDH using lactate, NAD^+, phenazine methosulphate and p-iodonitrotetrazolium violet.



0.2M Tris-HCl, pH8.2 and 100µl was added to each well, left for 15 min at 23°C. The reaction was terminated by the addition of isopropanol, containing 4% (v/v) 1.0M HCl (50µl). The plates were read immediately at 495nm in a Titertek Multiskan MCC/340 plate reader.

2.11.4.2. Cytotoxic effects of myasthenic serum and GPC on TE671 cells.

TE671 cells were harvested and replated as previously described.

Quadruplicate cell cultures in 96-well plates were washed with HBSS (2 x 50µl). After washing, fresh HBSS was added to each well (80µl), followed by the addition of myasthenic serum (10µl per well) and finally GPC (10µl). The cultures were then incubated at 37°C in an atmosphere of 10% CO_2/air for 3h. After this time the medium above the cells was transferred to a clean 96-well plate and assayed for LDH presence as previously described.

Control cultures, to which either normal human serum, no serum or complement, or each component alone was included in each test. Cytotoxicity was expressed as the percentage LDH released from test sera with or without complement, with respect to LDH released from Triton X-100 lysed cells, also included in the test.

2.11.4.3. Selective inhibition of endogenous serum LDH.

a) Using DEAE Sephadex A-50.

DEAE Sephadex A-50 (1g) was suspended in 20mM sodium phosphate buffer, pH6.8, (50ml), mixed thoroughly and allowed to sediment for 45 min at 23°C, after which time the buffer was decanted from the sedimented material. The suspension and decanting procedure was repeated three times, the resulting uniformly fine sediment was resuspended in buffer to give a final volume of 50ml.

DEAE Sephadex A-50 (1ml) was then mixed with serum (1ml) for 10min 23°C. This suspension was centrifuged (3000 rpm, 5 min,23°C) and the supernatant was retained for testing in cytotoxicity assays and RIA for anti-AChR antibody titre.

b) Using Matrex Blue-gel.

Serum samples were applied to a mini-column containing Matrix Blue-gel (1ml) and recirculated for 1 h at 23°C, after which, the eluant was retained for cytotoxity tests and RIA, as described above.

2.11.5. Determination of uptake of a) L-[Me-^{3}H] carnitine hydrochloride and b) [^{51}Cr] by TE671 cells.

TE671 cells were harvested and replated as

previously described. L-[Me-^{3}H] carnitine hydrochloride (specific activity 87µCi/mmol, radioactive concentration, 1mCi/ml) or [^{51}Cr] (radioactive concentration, 1mCi/ml) were diluted under sterile conditions in growth medium, before addition to 4 replicate culture wells (0-10µM, carnitine; 0-0.2µCi/ml,** chromium).

After incubation (18h, 37°C in an atmosphere of 10% CO_2/air), the cultures were washed (3x0.5ml growth medium), immediately extracted in 0.1M NaOH (2x0.5ml) and mixed with scintillation fluid (Optiphase safe, 5ml). The radioactivity of each culture well was determined by counting in a Packard Tricarb liquid scintillation counter.

** later increased (0-20µCi/ml).

2.11.5.1. Cytotoxicity of serum and complement on TE671 cells in culture.

Quadruplicate wells of TE671 cells were labelled by incubation (18h, 37°C) with [^{51}Cr] (2.5µCi/well), in an atmosphere of 10% CO_2/air. Cultures were then washed with growth medium (3x0.5ml, 2-3 min) and fresh medium (0.5ml) was added to each well, followed by the addition of heat inactivated serum +/- guinea pig complement. The cultures were then further incubated at 37°C for 3h. After incubation, cultures were washed with growth

medium (3x0.5ml, 2-3min), extracted with 0.1M NaOH (2x 0.5ml), mixed with scintillation fluid (Optiphase safe) and counted for radioactivity as previously described.

Control cultures to which no addition was made were included in the test. Cytotoxicity was expressed as the percentage loss of radio activity compared with controls, according to the formula:-

$$\frac{CRC - CRT}{CRC} \times 100\%$$

where CRC = counts retained by controls.

CRT = counts retained by test.

RESULTS.

3.1. Human skeletal muscle cells in culture.

3.1.1. Preparation of cultures.

Single cell suspensions were prepared from human fetal limbs as described in Section 2.10.3. Dissociation of tissue with 0.25% (w/v) trypsin for 1h at 37°C resulted in cell yields of 1.0-1.8x10^6 cells/ limb. Subsequent dissociation, employing additionally 0.25% (w/v) collagenase, resulted in slightly improved yields 0.5-3.5x10^6 cells/limb (Table 5), the latter procedure was routinely used. Cell viability, as judged by the exclusion of trypan blue dye, was greater than 95%.

As human muscle cells in culture were very susceptible to bacterial and fungal infection, reflecting the conditions involved in collection of the fetuses, limbs were routinely collected and stored in growth medium containing penicillin and streptomycin (10000U/ml) and fungizone (2.5µg/ml). Of 16 human muscle culture preparations, only five were viable for subsequent assay.

3.1.2. Morphology of cultures.

After several hours in culture, most of the cells were attached to the collagen substratum. The mononucleated cells were initially round in shape. After

Table 5. Cell yields after enzymatic and mechanical dissociation of human fetal skeletal muscle.

Culture	Dissociation	Cells/limb ($\times 10^6$)	Viability
1	trypsin	1.80	no
2	"	1.72	no
3	"	1.12	no
4	"	1.06	yes
5	trypsin/ collagenase	1.65	yes
6	"	1.80	no
7	"	1.23	yes
8	"	1.00	yes
9	"	0.80	no
10	"	0.50	no
11	"	3.50	no
12	"	1.80	no
13	"	2.62	no
14	"	1.90	no
15	"	2.00	yes
16	"	2.20	no

1 - 2 days in culture, spindle-shaped, bipolar cells could be distinguished from flat, multipolar cells. The morphology of the former is attributed to cells of myogenic origin and that of the latter is typical of fibroblasts.

The cultures then entered a period of rapid cell fusion resulting in the formation of a network of rapidly growing multinucleated fibres or myotubes (Fig. 14).

3.1.3. Inhibition of fibroblast growth.

After 3-4 days in culture, when fusion of myoblasts was judged morphologically to be complete, a mitotic inhibitor, cytosine arabinoside (10µM, final concentration) was added to reduce fibroblast growth. Cytosine arabinoside did reduce the fibroblast growth but also resulted in poor myotube growth.

3.1.4. Myotoxicity studies: Effect of serum and complement on human muscle cultures.

A preliminary myotoxicity study investigated the effects of heat inactivated (56°C, 1h) pooled normal human serum and a myasthenic serum sample (stored before use at -20°C for varying lengths of time) on human myotubes in culture.

Cultures in 24-well plates were labelled with L-[Me-^{3}H] carnitine as described (Section 2.10.4.), before

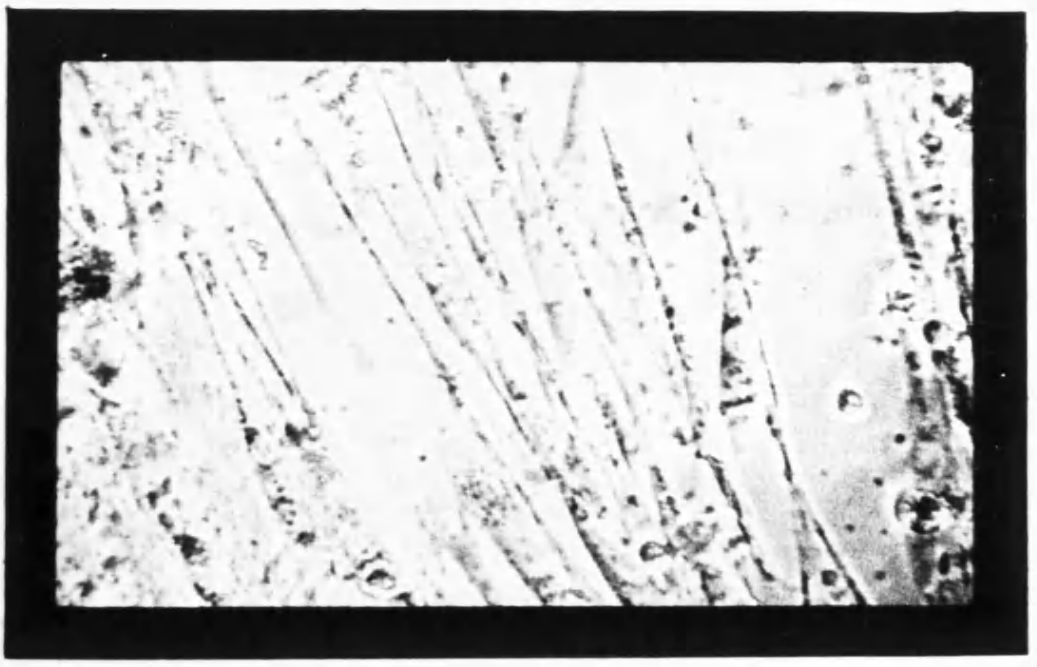
Fig. 14

LIGHT PHOTOMICROGRAPHS OF HUMAN MUSCLE CELLS, DAY 6 IN CULTURE

A)

X 40 MAGNIFICATION

B)



X 80 MAGNIFICATION

use in the myotoxicity assay. By dividing the 24 well plate into six groups of quadruplicate tests (Fig. 15), labelled cultures were incubated with normal or myasthenic serum (80µl, 12% v/v) and/or GPC (80µl, 12% v/v) for 3h at 37°C. Myotoxicity was calculated as previously described (Section 2.10.4.). The myotoxicity given by pooled normal human serum was -4.25%, a corresponding value for myasthenic serum was -3.5%. Values for myotoxicity for serum with complement gave 4.65% for pooled normal human serum and 6.04% for myasthenic serum.

In view of this apparent lack of relative toxicity of myasthenic serum, carnitine labelled cultures were incubated with increasing concentrations of heat inactivated serum (50 - 300µl, 3.3 - 20% v/v) in the presence of a constant concentration of GPC (300µl, 20% v/v), or alternatively with increasing concentrations of GPC (50 - 300µl, 3.3 - 20% v/v) in the presence of a constant concentration of serum (300µl, 20% v/v). From Fig. 16 it can be seen that myotoxicity increased with increasing concentrations of myasthenic serum or GPC. Normal human serum had little effect in these experiments (myotoxicity <10%).

In further myotoxicity assays, human muscle cultures, labelled with L-[Me-^{3}H] carnitine as described above, were incubated with an increased concentration of both serum and GPC (300µl, 20% v/v). Using these conditions, the complement-mediated myotoxicities of pooled normal human serum and of 5 myasthenic patients

FIG. 15 Arrangement of myotoxicity test samples for 6 tests on a 24 well culture plate.

1	3	5	6	2	4
1	3	5	6	2	4
2	4	6	1	3	5
2	4	6	1	3	5

Where 1 = no addition
2 = normal human serum
3 = " " " and GPC
4 = myasthenia gravis serum
5 = " " " and GPC
6 = GPC

Fig. 16. Myotoxicity of myasthenic serum on human muscle cultures.

a) effect of increasing serum concentration.

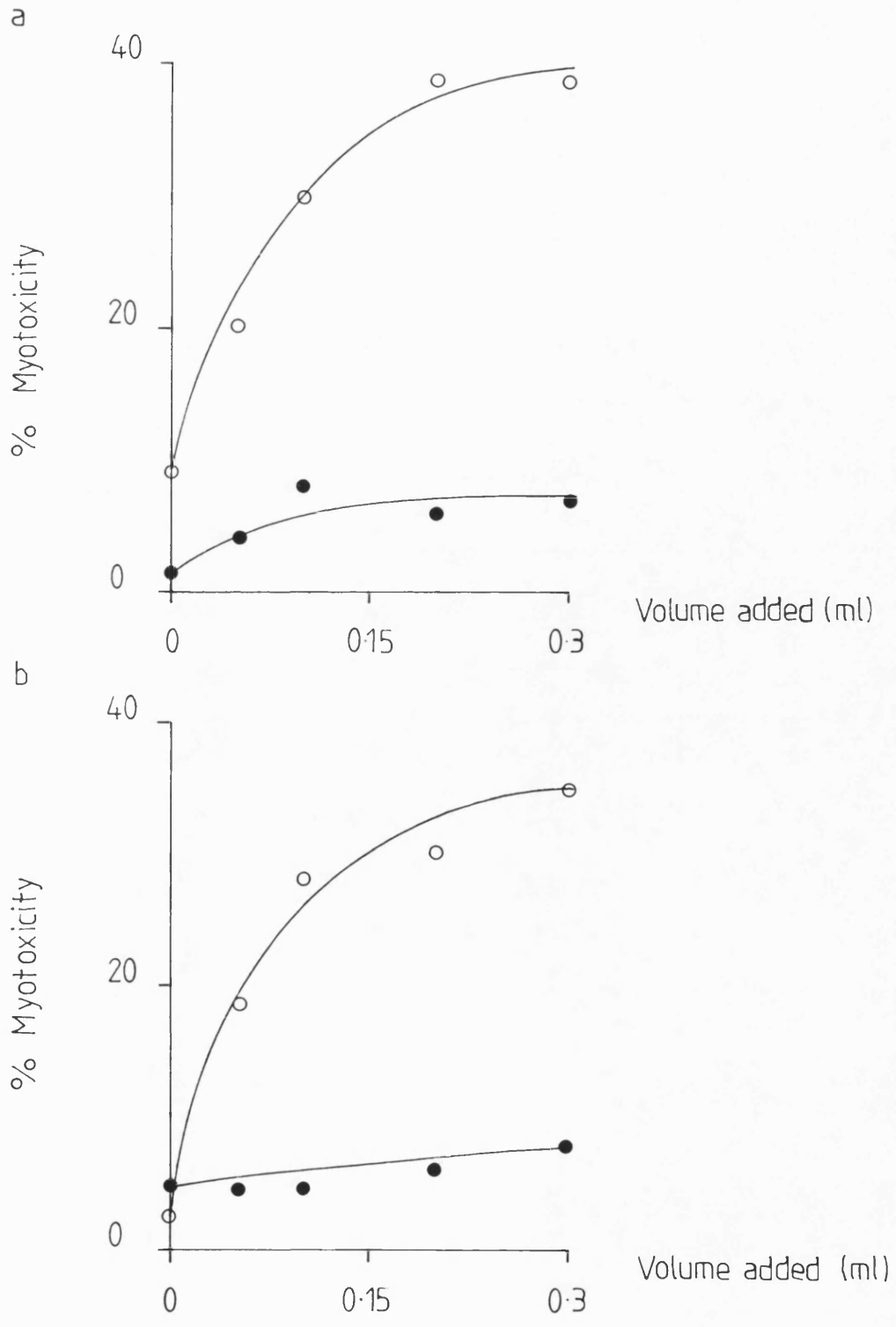
b)effect of increasing GPC concentration.

a) Seven day old human muscle cultures labelled with L-[Me-^{3}H] carnitine were exposed to increasing concentrations of normal human serum (●) or a myasthenic serum (O), in the presence of GPC (0.3ml).

b) Seven day old human muscle cultures labelled with L-[Me-^{3}H] carnitine were exposed to increasing concentrations of GPC in the presence of a fixed concentration (0.3ml) of normal human serum (●) or myasthenic serum (O).

Myotoxicity was calculated as previously described (Section 2.10.4.). Results are from a representative experiment.

FIG. 16 Myotoxicity of myasthenic serum on human muscle cultures.



were assessed (Fig. 17). The measured myotoxicity given by heat inactivated pooled normal human serum was in the range -14 -- 8% (-2.8 ± 4.2% ; mean ± SEM (n=5)). Corresponding values given by heat inactivated myasthenic serum samples were in the range -10 -- 30% (5 ± 7%; mean ± SEM (n=5)). Values for myotoxicity for serum with complement were 11 -- 31% (17 ± 4%; mean ± SEM (n=5)) for pooled normal human serum and 29 -- 49% (35 ± 3%; mean ± SEM (n=5)) for myasthenic serum samples. Myotoxicity of GPC alone was 1 -- 30% (16 ± 5%; mean ± SEM (n=5)). The results for each individual myasthenic serum sample are shown in Fig. 17, where it can be seen that the combination of myasthenic serum and GPC gave higher myotoxicity values than for pooled normal human serum in the presence or absence of GPC, myasthenic serum alone or GPC alone. The measured complement-mediated myotoxicity of myasthenic sera did not correlate with their anti AChR antibody titre.

The results indicated a complement-mediated lysis of cultured human muscle cells by myasthenic serum. In view of the spread of myotoxicity values obtained (Fig. 17) there was clearly a need for many more assays, but owing to the erratic supply of cells and the freqency with which contamination occurred, this was not practicable. Accordingly, an alternative supply of human cells expressing AChR was sought.

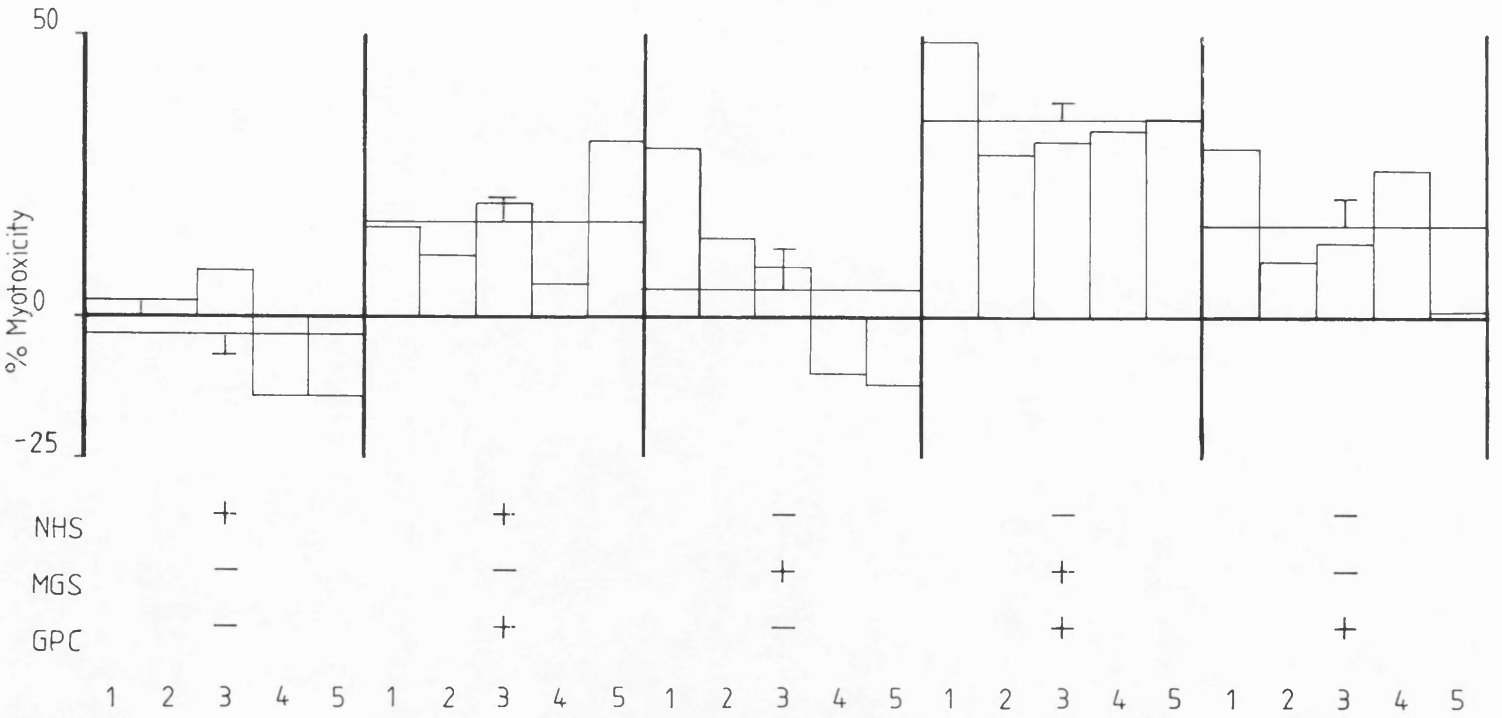
Fig. 17. Myotoxicity of serum and GPC on human muscle cultures.

Seven day old human muscle cultures grown in Dulbecco's modified Eagle's medium, were labelled with L-[Me-^{3}H] carnitine as previously described (Section 2.10.4.) and exposed to samples of heat inactivated test serum (300µl, 20% v/v) in the presence of GPC (300µl, 20% v/v) for 3h at 37°C. Cultures to which no additions were made (control) or to which GPC alone (300µl, 20% v/v) was added were run simultaneously. At the end of the incubation time, the cultures were washed and solubilised for counting. Myotoxicity was calculated as described (Section 2.10.4.) by comparison with control cultures.

Results are the means for five different myasthenic sera tested in quadruplicate culture wells. The mean ± SEM for each group of tests is shown.

MG patient	anti-AChR antibody titre (nM).
MG1	42.00
MG2	20.60
MG3	71.30
MG4	10.90
MG5	0.26

FIG. 17 Myotoxicity of serum and complement on human muscle cultures.



3.2. TE671 CELL CULTURE.

3.2.1. Cellular morphology and growth characteristics.

In culture, TE671 cells consist mainly of polygonal or fusiform cells having two, three or four short unbranched processes and multiple nuclei (Fig. 18 and Table 2, Section 1.4.4.1.). In most passages, the most frequent cell type was the spindle-shaped cell. THe round and bipolar cells were sometimes difficult to distinguish from the other cell types, suggesting that these may be different forms of the same cell. TE671 cells exhibited independent cell growth on either a solid substrate (in 80cm^3 flasks, in continuous culture) or chemical substrate (polylysine coated culture plates) and were not contact inhibited, continuing to grow over an already established cell monolayer.

3.2.2. Characterisation of TE671 cell AChR.

3.2.2.1. AChR content of intact and solubilised TE671 cells.

The extents of [^{125}I] α-BGT binding to intact cells and to those cells solubilised with the non-ionic detergent, Triton X-100 are shown in Table 6. As cells are known to be impermeable to α-BGT (Kemp and Edge, 1987), incubation in the absence of detergents would be

Fig. 18. Light photomicrographs of TE671 cells in culture.

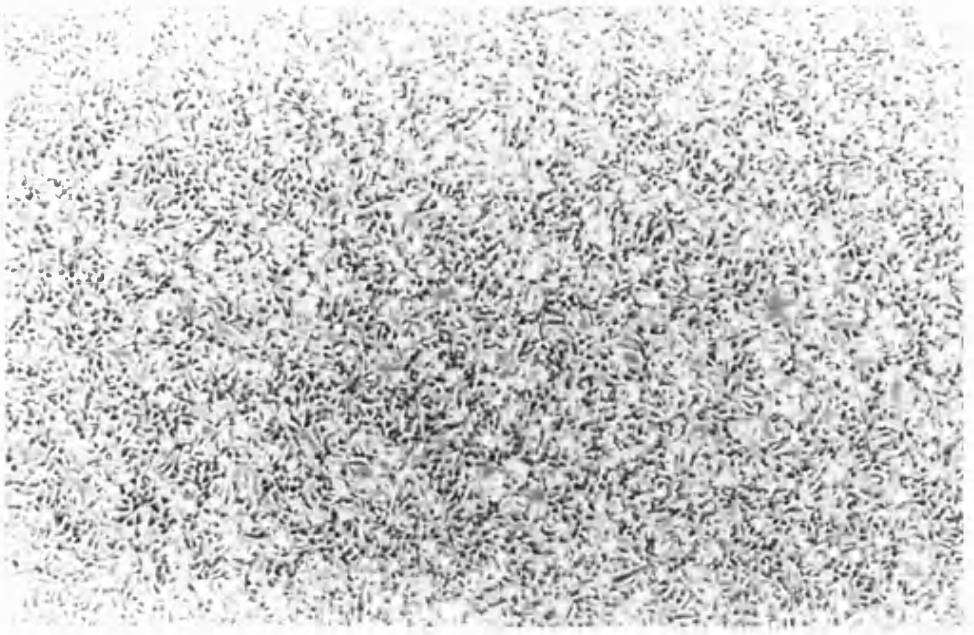
a) x40 magnification

b) x100 magnification

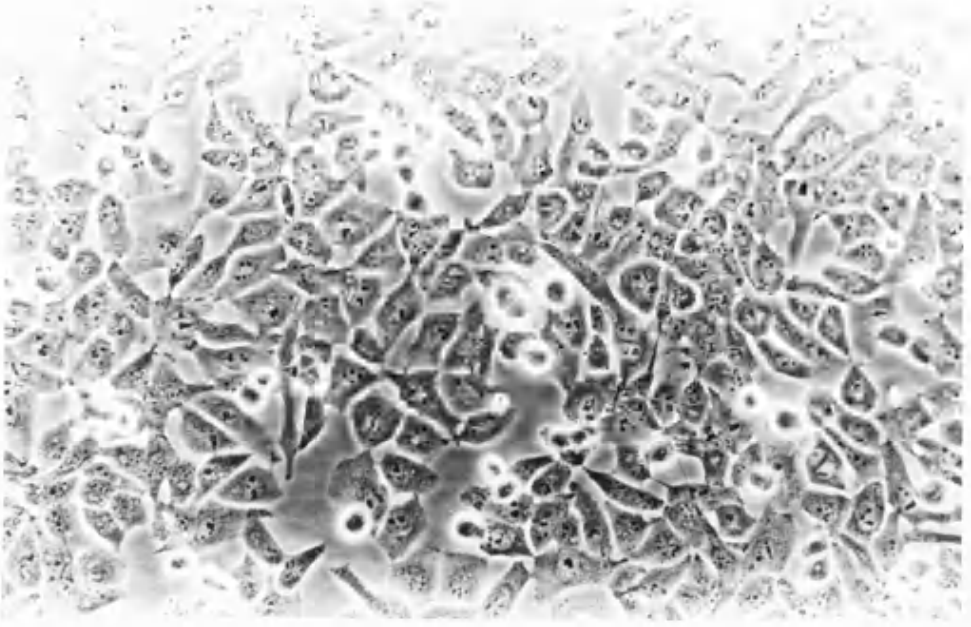
c) x400 magnification

FIG. 18

a)



b)



c)

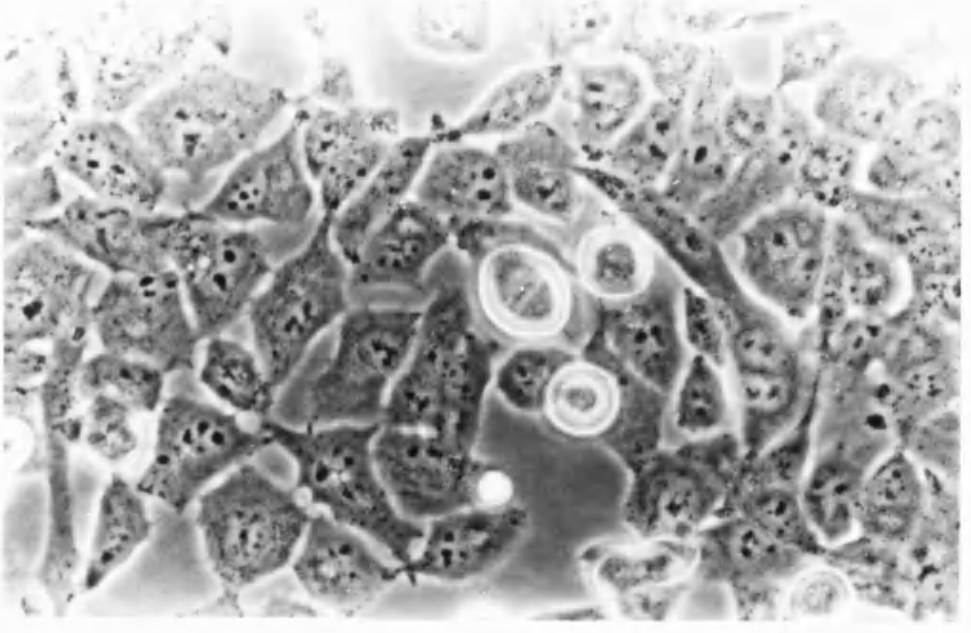


Table 6. [^{125}I] α-BGT binding to intact and solubilised TE671 cells.

Binding was determined as previously described (Section 2.11.3.2.), using the cholinergic antagonists d-tubocurarine (1.0mM) or α-BGT (1.0μM) to determine non-specific binding.

Results are mean +/- SEM (n=3), with the assay carried out in triplicate.

Table 6. [^{125}I] α-BGT binding to intact and solubilised TE671 cells.

	AChR (pmol/mg protein	
Ligand	Intact	Solubilised
d-TC	30.85 ± 5.99	52.05 ± 10.63
α-BGT	26.21 ± 1.90	47.71 ± 4.49

expected to saturate only cell surface sites. If additional sites are present, then solubilisation of the cells with detergent would result in their being detected. The approximately two-fold increase in α-BGT binding sites observed on solubilisation suggests that a significant fraction of the α-BGT binding sites on TE671 cells are intracellular.

3.2.2.2. Properties of [^{125}I] α-BGT binding to TE671 cells.

[^{125}I] α-BGT bound to a single saturable high affinity site on intact TE671 cells as indicated by linear Scatchard plots (Fig. 19).

The Kd for binding to intact cells (mean ± SEM (n)) was 2.03 ± 0.41nM (5). The Bmax was calculated (mean ± SEM (n)) as 24.13 ± 3.70 fmol/mg protein (5).

3.2.2.3. Apparent constants for inhibition, by cholinergic ligands, of [^{125}I] α-BGT binding to TE671 cells.

Competition binding assays using cholinergic ligands to inhibit high affinity [^{125}I] α-BGT binding to TE671 cells were carried out as described (Section 2.11.3.4.). Dose response curves were generated (Figs. 20 and 21) from which it can be seen that the antagonist α-BGT was the most potent inhibitor of [^{125}I] α-BGT binding (Tables 7 and 8), being two orders of

Fig. 19. Binding of [^{125}I] α-BGT to TE671 cells.

The binding of [^{125}I] α-BGT to triplicate culture wells of confluent TE671 cells was determined as described (Section 2.11.3.3.).

Binding data are from 1 representative experiment showing:

(○) Binding in the absence of d-tubocurarine chloride (total binding).

(●) Binding in the presence of d-tubocurarine chloride (non-specific binding).

(△) Specific binding.

Inset: Scatchard analysis of the binding data in Fig. 19.

FIG. 19 Binding of [^{125}I] α-BGT to TE671 cells in culture.

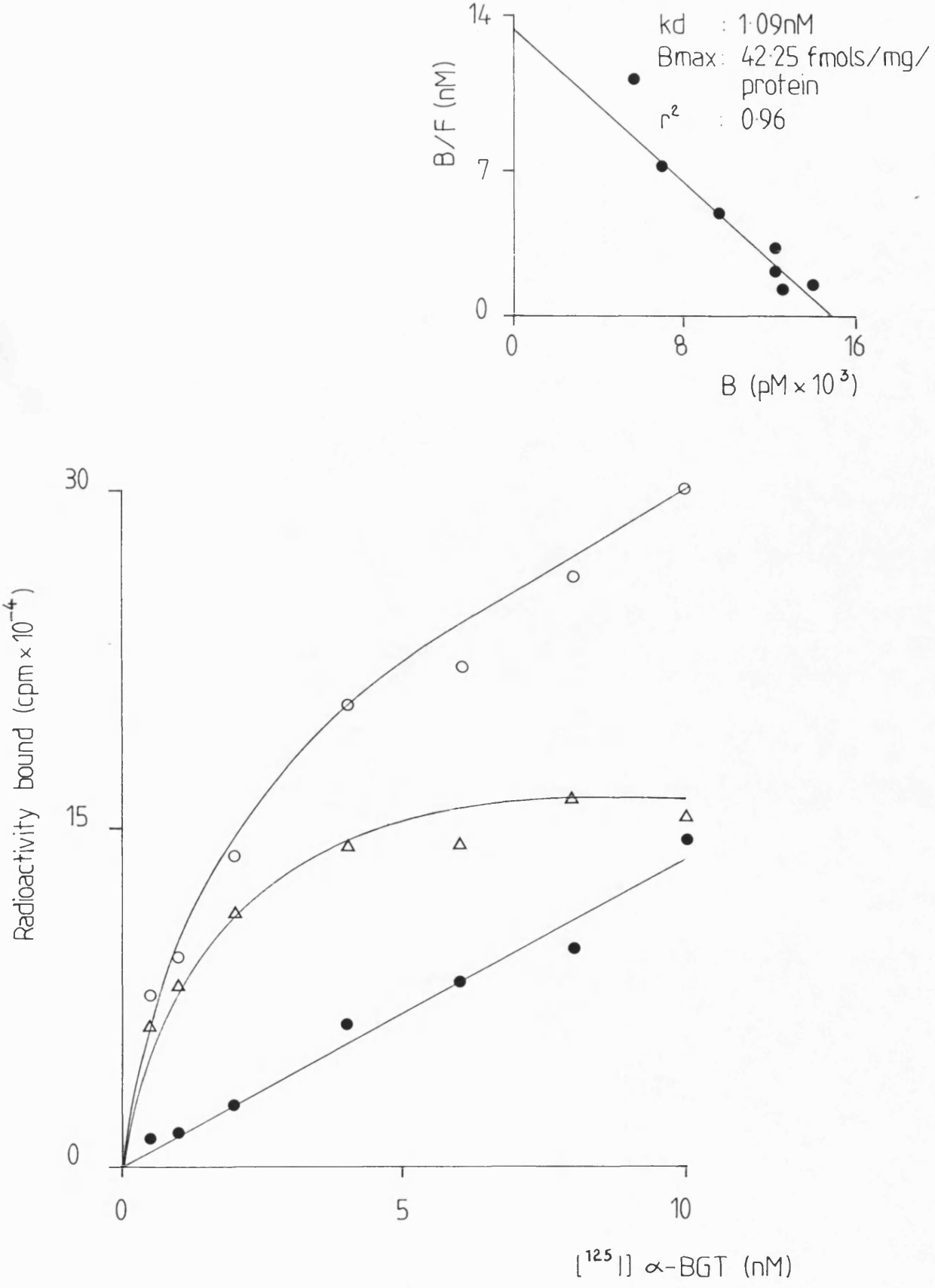


Fig. 20. Competition binding assays with cholinergic antagonists for [^{125}I] α-BGT binding to TE671 cells.

Competition assays as described in Section 2.11.3.4., for [^{125}I] α-BGT binding to TE671 cells were carried out in the presence of α-BGT (△), d-tubocurarine (●), decamethonium (▲), benzoquinonium (○) and MLA (■). Results with the exception of α-BGT are the mean ± of 3 experiments, with each ligand concentration assayed in triplicate.

Table 7. Inhibition of [^{125}I] α-BGT binding to TE671 cells by cholinergic antagonists.

Competition assays were performed as described (Section 2.11.3.4.), using [^{125}I] α-BGT at 2.0nM. IC50 values were derived from linear transformations of dose-response curves; Ki values were derived from IC50 values (Cheng and Prusoff, 1973), assuming Kd values for [^{125}I] α-BGT binding to TE671 cells of 2.0nM. Data are the mean of 2 or 3 of three independent determinations, carried out in triplicate.

FIG. 20 Competition binding assays with cholinergic antagonists for [^{125}I] α-BGT binding to TE671 cells.

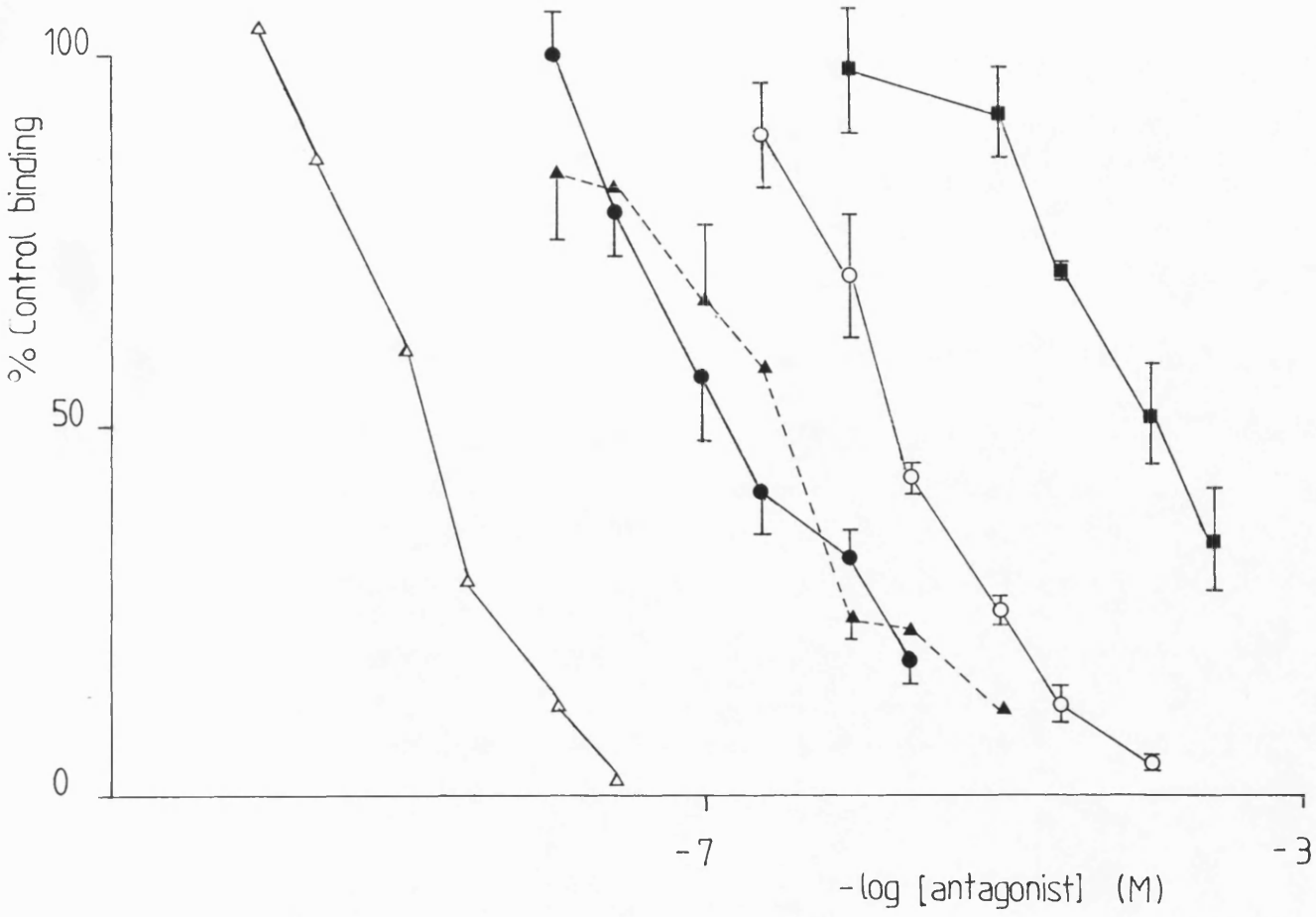


Table 7. Inhibition of [^{125}I] α-BGT binding to TE671 cells by cholinergic antagonists.

	α-BGT (△)	d-TC (●)	BZQ (○)	MLA (■)	DBr (▲)
IC50	1.3×10^{-9}M	1.9×10^{-7}M	2.5×10^{-6}M	1.0×10^{-4}M	2.8×10^{-7}M
Ki	6.5×10^{-10}M	9.8×10^{-8}M	1.3×10^{-6}M	5.0×10^{-5}M	1.4×10^{-7}M
n_B	1.21	0.60	0.88	0.87	0.76
r^2	0.99	0.98	0.99	0.98	0.98

Fig. 21. Competition assays with cholinergic agonists for [^{125}I] α-BGT binding to TE671 cells.

Competition assays as described in Section 2.11.3.4., for [^{125}I] α-BGT binding to TE671 cells were carried out in the presence of:

a) (-)-Nicotine (○) and acetylcholine (△).

b) Carbamylcholine (●) and (+)-nicotine(■).

Results are the mean ± SEM of three experiments, or the mean of two experiments (carbamylcholine), with each ligand concentration assayed in triplicate.

Table 8. Inhibition of [^{125}I] α-BGT binding to TE671 cells by cholinergic agonists.

Competition assays were performed as described (Section 2.11.3.4.) using [^{125}I] α-BGT at 2.0nM. IC50 values were derived from linear transformations of the dose response curves; Ki values were derived from IC50 values (Cheng and Prusoff, 1973), assuming Kd values for [^{125}I] α-BGT binding to TE671 cells of 2.0nM. Data are the means of at least three independent determinations, carried out in triplicate.

FIG. 21 Competition binding assays with cholinergic agonists for [^{125}I]αBGT binding to TE671 cells.

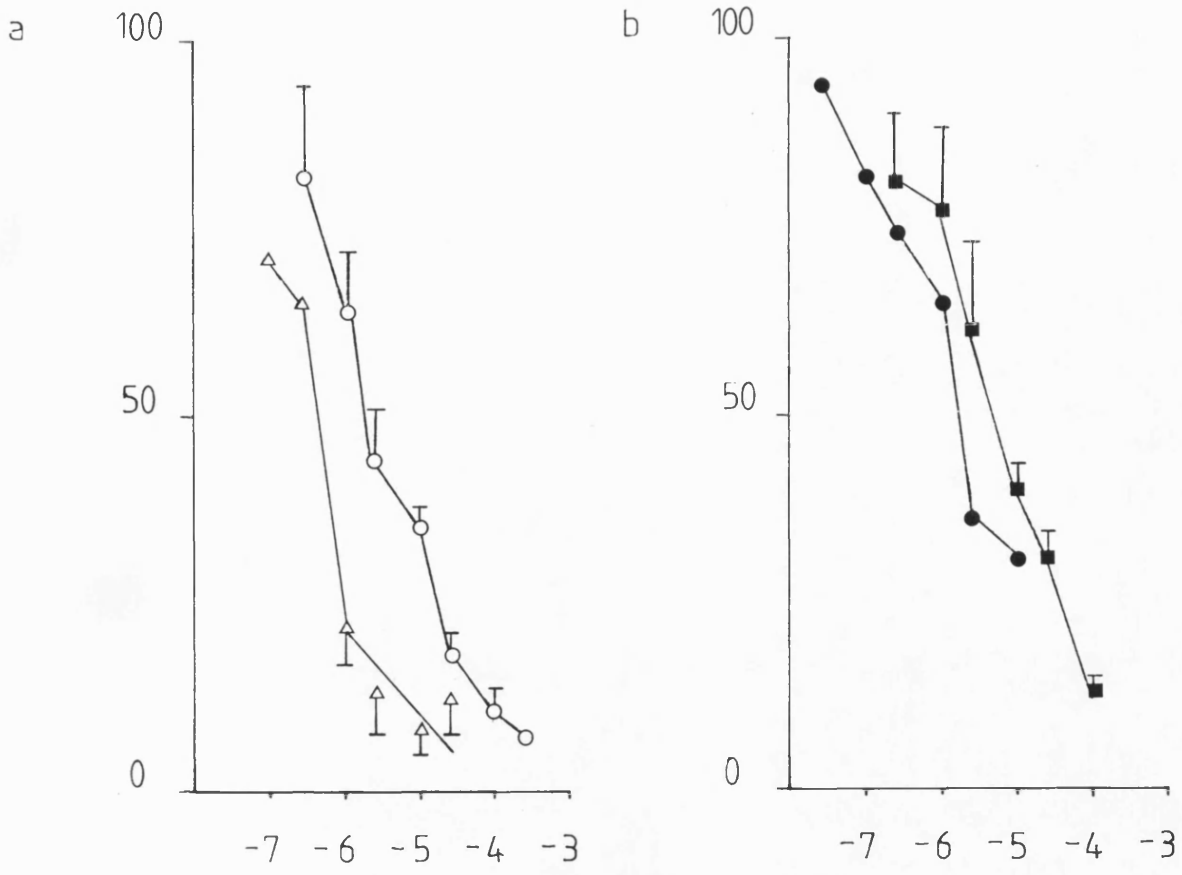


Table 8. Inhibition of [^{125}I] α-BGT binding to TE671 cells by cholinergic agonists.

	(-)Nic (○)	(+)Nic (■)	ACh (△)	Carb (●)
IC50	2.6x10^{-6}M	4.9x10^{-6}M	3.4x10^{-7}M	1.7x10^{-6}M
Ki	1.3x10^{-6}M	2.4x10^{-6}M	1.7x10^{-7}M	8.7x10^{-7}M
n_H	0.59	0.59	0.85	0.61
r^2	0.99	0.98	0.97	0.98

magnitude more potent than d-tubocurarine, decamethonium, benzoquinonium, the stereoisomers of nicotine and carbamylcholine. Methyllycaconitine (MLA) showed the lowest potency in these studies. The lack of effect of MLA was further investigated in the same assay but using frog muscle and human muscle preparations (Section 2.11.3.5.). From the results shown in fig. 22 and Table 9, it can be seen that MLA showed slightly higher potency in competing for high affinity [^{125}I] α-BGT binding compared with that in TE671 cells. These results contrast to its very potent effect in rat brain P2 membranes prepared as described (Section 2.5.). Other ligands, choline, hexamethonium, atropine, glutamine and glycine were also tested, but were without effect.

3.2.3. Inhibition of [^{125}I] α-BGT binding to TE671 cells by myasthenic serum.

Serum samples from 3 patients with myasthenia gravis were tested for their ability to inhibit high affinity [^{125}I] α-BGT binding to intact TE671 cells as previously described (Section 2.11.3.6.). The sera tested varied in their anti-AChR antibody titres as determined by radioimmunoassay (Section 2.6.). Hill plots were generated for each serum tested (Fig. 23) and IC50 values were obtained from them (Table 10). The results show a correlation between decreasing anti-AChR antibody titre and decreasing IC50 value.

Fig. 22. Competition binding assays with MLA for [^{125}I] α-BGT binding to various tissues.

Competition binding assays for [^{125}I] α-BGT binding to frog muscle extract (●), human muscle extract (○) and rat brain P2 membranes (□) were carried out in the presence of MLA (Section 2.11.3.5.). Results are the mean ± of 3 determinations (frog and rat brain P2 membranes) or 5 determinations (human), with each ligand concentration assayed in triplicate.

Table 9. Inhibition of [^{125}I] α-BGT binding to various tissue preparations by MLA.

Competition assays were performed as described (Section 2.11.3.5.), using [^{125}I] α-BGT at 2.0nM. IC50 values were derived from linear transformations of dose-response curves; Ki values were derived from IC50 values (Cheng and Prusoff, 1973), assuming Kd values of 1.0nM for α-BGT binding to both tissues. Data are the mean of at least 3 determinations carried out in triplicate.

FIG. 22 Competition binding assays with MLA for [^{125}I] α-BGT binding to various tissues.

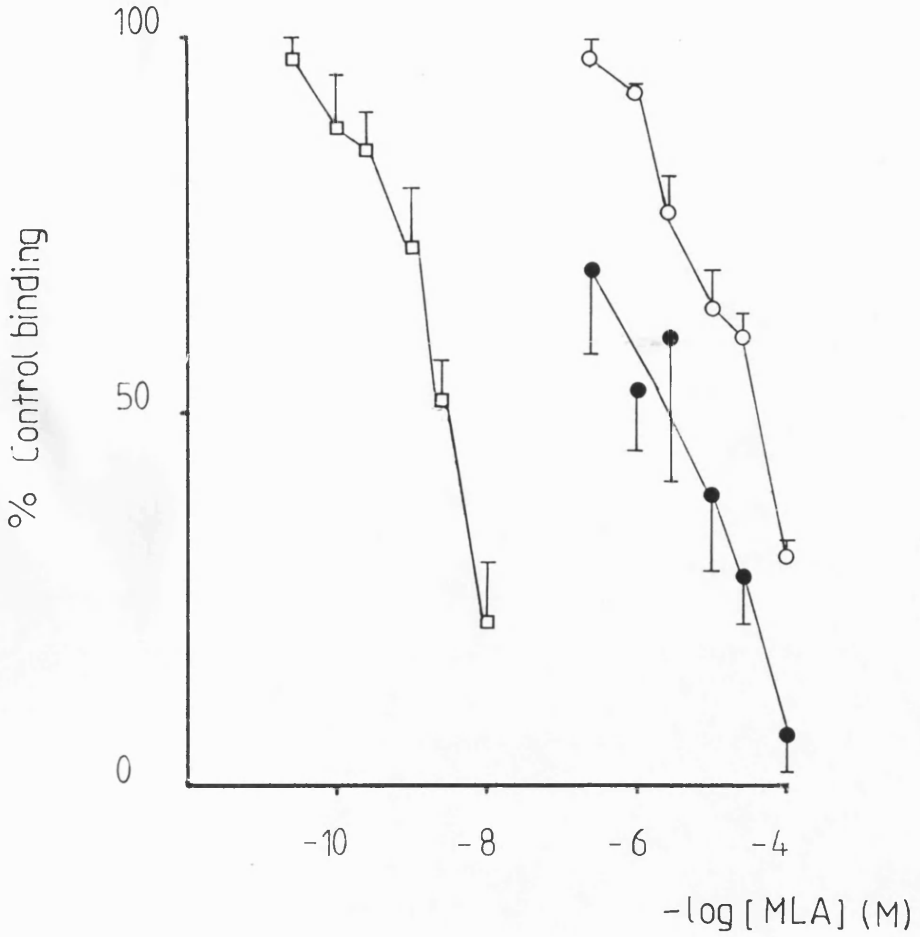


Table 9. Inhibition of [^{125}I] α-BGT binding to various tissues by MLA.

	Frog (●)	Human (○)	Rat brain (□)
IC50	2.4×10^{-6}M	2.9×10^{-5}M	2.2×10^{-9}M
Ki	1.2×10^{-6}M	1.4×10^{-5}M	1.1×10^{-9}M
n_H	0.60	0.63	0.75
r^2	0.93	0.98	0.76

Fig. 23. Inhibition of [^{125}I] α-BGT binding to TE671 cells by myasthenic serum.

High affinity [^{125}I] α-BGT binding to intact TE671 cells was inhibited following exposure of the cells to increasing concentrations of myasthenic serum samples (Section 2.11.3.6.).

Results are expressed as a percentage of binding in the presence of normal human serum (control binding) which was without effect.

The results shown are either the mean ± SEM of 3 experiments or the mean of 2 experiments.

(●) MG1

(□) MG2

(■) MG6

FIG. 23 Inhibition of [^{125}I] α-BGT binding to TE671 cells by myasthenic sera.

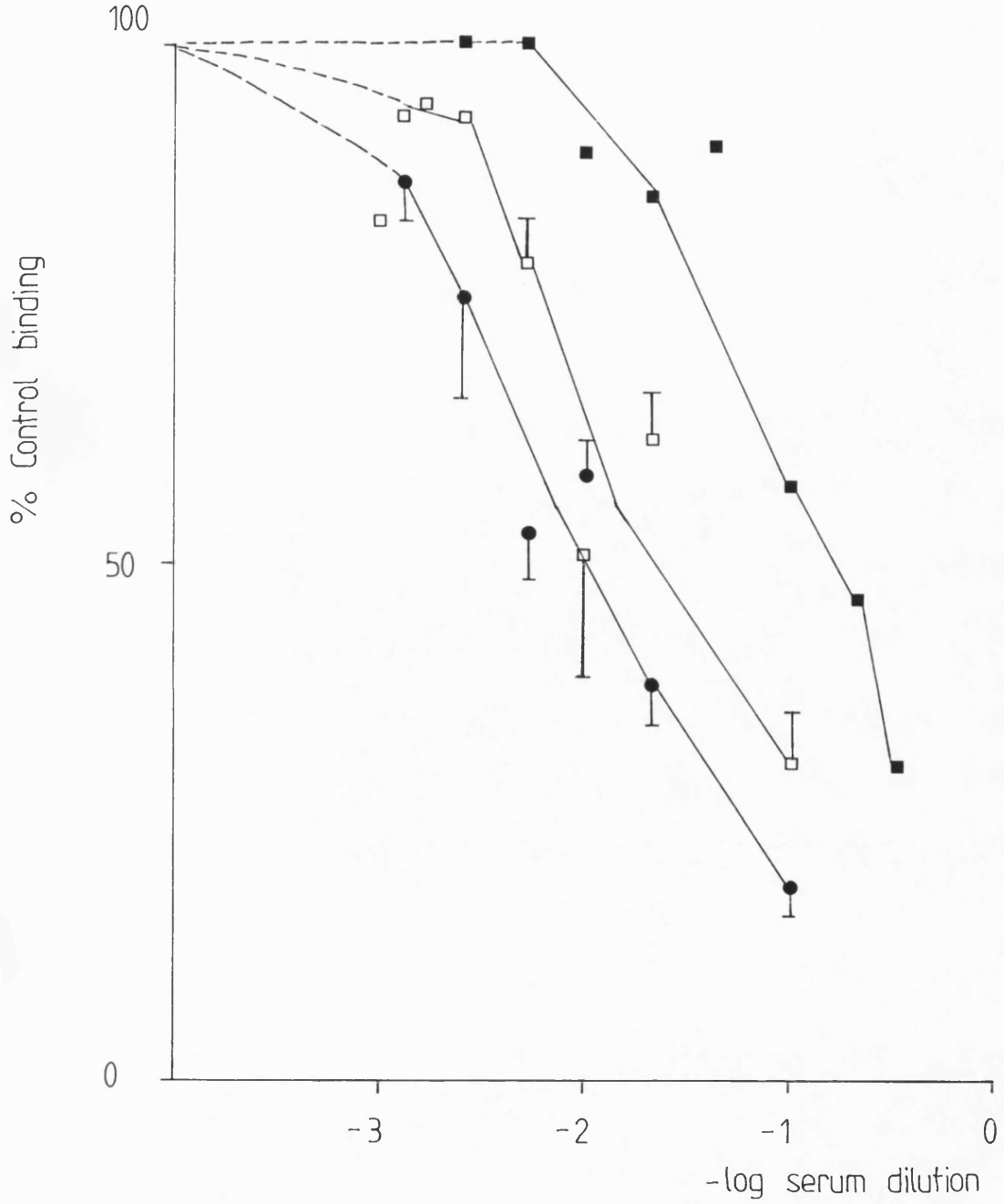


Table 10 Summary of results obtained from inhibition of [^{125}I] α-BGT binding by myasthenic sera.

Anti-AChR antibody titre (nM)		IC50 value (antiserum dilution) from Hill plot.
MG1	42.00	1/80
MG2	20.60	1/30
MG6	9.00	1/6

3.2.4. TE671 CELLS: CYTOTOXICITY STUDIES.

3.2.4.1. Lactate dehydrogenase [EC 1.1.1.27] in TE671 cells.

TE671 cells were harvested as previously described (Section 2.11.2.). The presence of LDH was detected by using a colorimetric assay based on that described by Bergmeyer and Bernt (1974) (for details, see Section 2.11.4.2.).

Triton lysis of TE671 cells demonstrated high levels of LDH as monitored by an increase in optical density at 492nm. The optical density at 492nm for seeding 5000 - 10000 cells/100µl/well was in the range 1.399 ± 0.028 - 2.478 ± 0.033 (mean ± SEM (n=8)). All subsequent experiments were carried out at seeding densities that resulted in cell numbers within this range at the time of assay.

3.2.4.2. Cytotoxic effects of serum and GPC on TE671 cells: Preliminary investigation.

TE671 cells were harvested, resuspended in HBSS and plated out into each well of a 96 well plate as previously described (Section 2.11.2.).

These cells were incubated, in quadruplicate, with heat-inactivated normal human serum samples or a myasthenic serum sample (10µl, 10% v/v) in the presence or absence of GPC (10µl, 10% v/v) for 3h at 37°C in an

atmosphere of 10% CO_2/air.

Cytotoxicity of serum with or without GPC was calculated as a percentage of LDH released from these cells with respect to LDH released from the same cells lysed with Triton X-100 as previously described (Section 2.11.4.2.).

Using the conditions described above, heat inactivated serum, both normal and myasthenic, with or without GPC, gave similar values of cytotoxicity (Table 11). This led to a further investigation of a larger pool of samples.

3.2.4.3. Extended study.

TE671 cells in 96 well plates were set up as described above (Section 3.2.4.2.). The effects of six heat inactivated normal human serum samples, a pooled normal human serum sample and four myasthenic serum samples (10µl, 10% v/v) were investigated on quadruplicate cultures in the presence or absence of GPC (10µl, 10% v/v). After incubation for 3h at 37°C in an atmosphere of 10% CO_2/air, cytotoxicity measured by release of LDH was calculated as previously described (Section 2.11.4.2.).

Heat inactivated normal human serum samples in the absence of GPC showed cytotoxicity values within the range 28-40% (mean 34%), in the presence of GPC, cytotoxicity values were very similar, within the range 30-41% (mean 36%). Heat inactivated myasthenic serum

Table 11. Cytotoxicity of serum and complement on TE671 cells as monitored by release of cytosolic LDH.

TE671 cells in a 96 well plate were exposed to samples of test serum (10µl, 10% v/v) in the presence or absence of GPC (10µl, 10% v/v) for 3h at 37°C. Control cultures to which no additions were made and those to which GPC alone was added were run simultaneously. At the end of the incubation period, the cultures were tested for release of cytosolic LDH as described (Section 2.11.4.2.) as a percentage of LDH released from cells lysed with Triton X-100. Each serum sample was added to quadruplicate culture wells.

Table 11. Cytotoxicity of serum and complement on TE671 cells, monitored by release of cytosolic LDH.

Treatment	Anti-AChR Ab titre (nM)	Optical Density 490nm ± (SEM) (n=4)	LDH Released % Triton lysed cells (cytotoxicity)
Triton-lysed	-	2.27±0.42	100
HBSS no cells	-	0.00	0
HBSS with cells	-	0.097±0.008	4
NHS (1)	-	0.641±0.037	28
NHS (1) + GPC	-	0.661±0.031	29
NHS (2)	-	0.738±0.032	34
NHS (2) + GPC	-	0.806±0.029	35
MG1	42.00	0.701±0.054	31
MG1 +GPC	42.00	0.785±0.062	34
GPC	-	0.397±0.028	17

NHS = Normal human serum
MG = Myasthenia gravis serum
GPC = Guinea pig complement

samples showed cytotoxicity values essentially no different to those observed with normal human serum; values within the range 25-38% (mean 31%) in the absence of GPC and 27-38% (mean 33%) in the presence of GPC (Fig. 24), indicating a lack of discrimination between the groups of serum samples under these conditions.

3.2.4.4. Titration of serum.

Quadruplicate cultures of TE671 cells in 96 well plates were set up as previously described (Section 2.11.2.). The effects of increasing concentrations of four different heat inactivated myasthenic serum samples (0 - 10µl, 0 - 10% v/v) were tested in the presence or absence of a fixed concentration of GPC (10µl, 10% v/v). A heat inactivated pooled normal human serum was tested under the same conditions in parallel experiments. Cytotoxicity, measured by release of LDH was calculated as previously described (Section 2.11.4.2.).

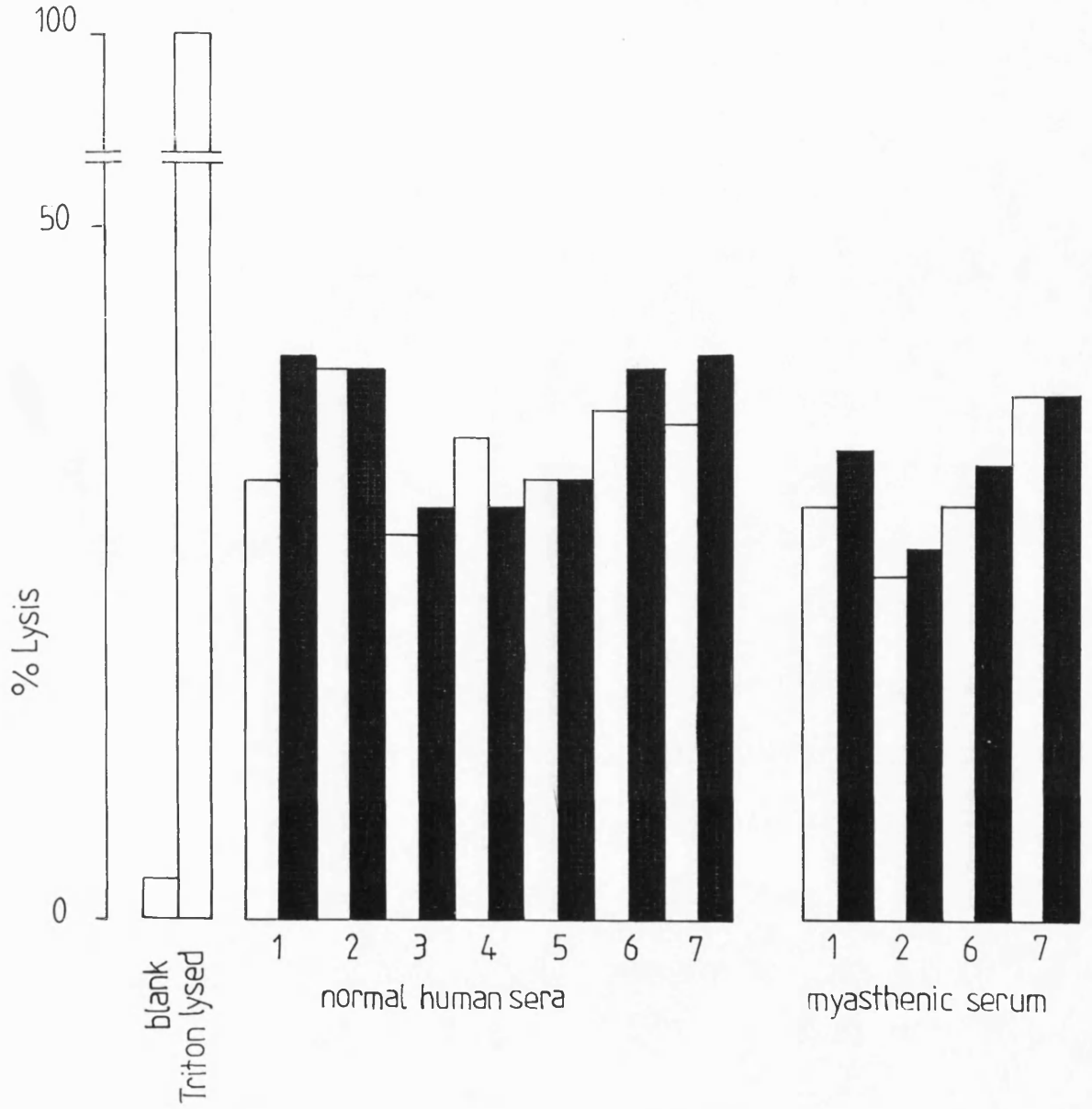
The results demonstrate an increase in cytotoxicity as the concentration of serum increased in both the myasthenic serum samples and the pooled normal human serum from the corresponding experiment (Fig. 25a and b). This effect was mimicked in the presence of complement (Fig. 25c and d). Whilst the results showed variations between individual experiments (Fig. 25a,b,c and d), there was little difference in the values of cytotoxicity between pooled normal human serum and

Fig. 24. Cytotoxicity of serum and complement on TE671 cells as monotored by release of cytosolic LDH.

TE671 cells in 96 well plates were exposed to samples of heat inactivated normal and myasthenic serum samples (10µl, 10% v/v) in the presence (solid bars) or absence (open bars) of GPC (10µl, 10% v/v). Control cultures to which no additions were made were included in the test.

Cytotoxicity was calculated as previously described. Results are the mean of two experiments.

FIG. 24 Cytotoxicity of serum and complement on TE671 cells as monitored by release of cytosolic LDH



anti-AChR antibody titre (nM)	-	-	-	-	-	-	-	42·0	20·6	9·0	76·1

Fig. 25. Cytotoxic effect of increasing concentration of normal or myasthenic sera, monitored by release of cytosolic LDH.

TE671 cells in 96 well plates were set up as previously described (Section 2.11.2.) and exposed to pooled normal human serum or four different myasthenic sera (0 - 10µl, 0 - 10 % v/v) in the presence or absence of GPC (10µl, 10% v/v). Cytotoxicity was calculated as the percentage LDH released compared with cells lysed with Triton X-100 (Section 2.11.4.2.).

a) Increasing concentrations of normal human serum without GPC. (□), (■), (●), (○) and (△) corresponding MG patients (b).

b) Increasing concentrations of 4 myasthenic serum samples without GPC. (○ :MG3), (■ :MG9), (□ :MG8) and (● :MG1).

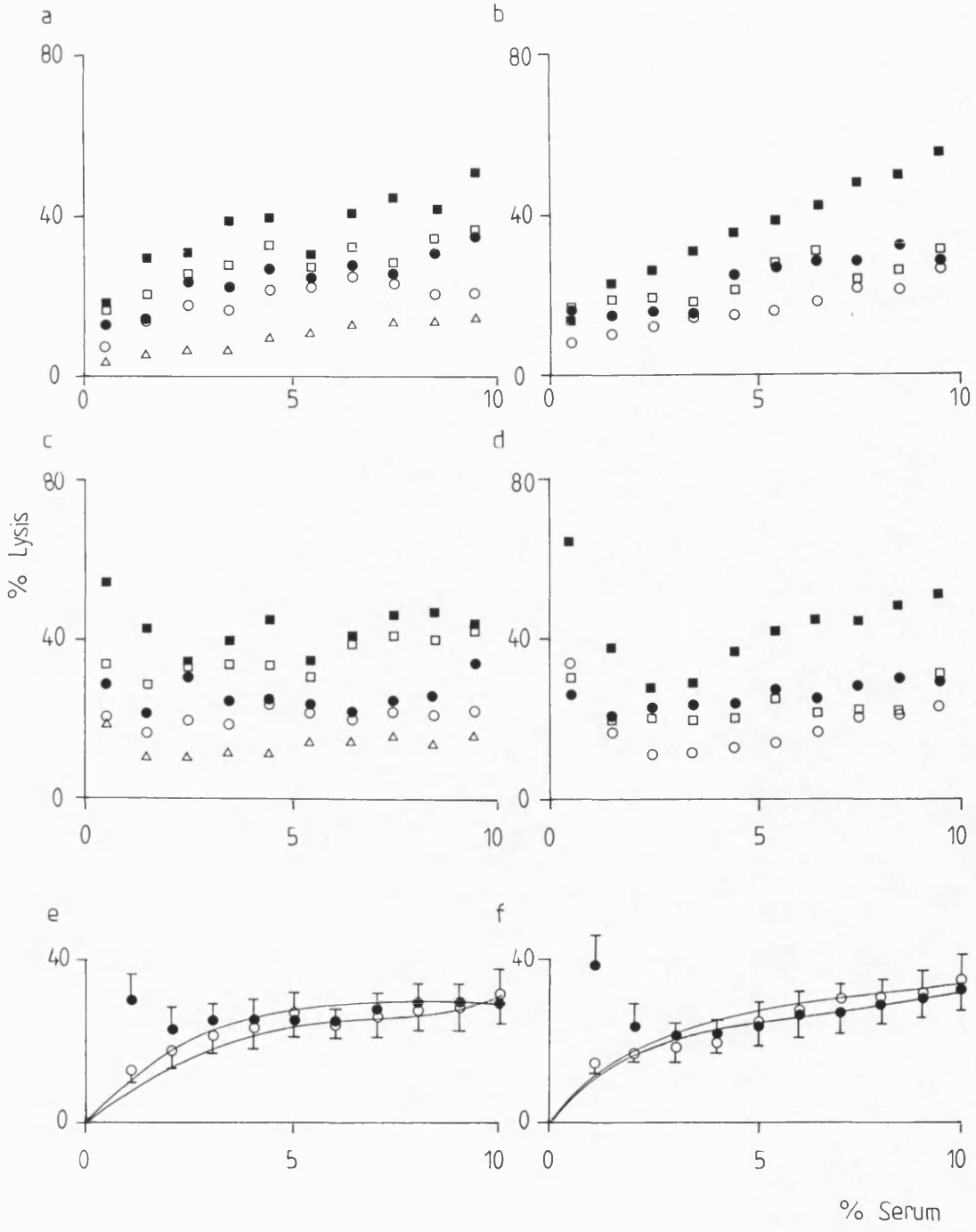
c)Increasing concentrations of normal human serum in the presence of GPC. Symbol details as above ("a").

d)Increasing concentrations of 4 myasthenic serum samples in the presence of GPC. Symbol details as above ("b").

d)Pooled results of increasing concentrations of normal human serum in the absence (○) or presence (●) of GPC. Results are the mean ± SEM, n=5.

f)Pooled results of 4 myasthenic patients in the absence of GPC (○) or presence (●) of GPC. Results are the mean ± SEM, n=4.

FIG. 25 Cytotoxic effect of increasing concentration of normal or myasthenic sera, monitored by release of cytosolic LDH.



myasthenic serum in the presence or absence of GPC (Fig. 25e and f). However, an increase in cytotoxicity in the presence of GPC was noticeable after the addition of 1µl of serum in the test and control samples (Fig. 25e and f), this effect was further investigated (Section 3.2.4.6.).

As the myasthenic serum samples used in this study had been stored, frozen for a considerable period of time, a cytotoxic effect of fresh myasthenic serum samples was also investigated.

3.2.4.5. Titration of fresh myasthenic serum.

Fresh serum samples from myasthenic patients were obtained from Southmead Hospital, Bristol, U.K.. In the case of large volumes of serum (>200ml), half of the serum was heat inactivated (56°C) and the remainder untreated. The serum was then stored, frozen, in 1ml and 2ml samples. Samples were thawed as required for each experiment and any serum remaining was discarded.

Quadruplicate cultures of TE671 cells were set up as previously described (Section 2.11.2.). Fresh myasthenic serum samples were titrated as described above (0-10µl, 0-10% v/v) in the presence or absence of a fixed concentration of GPC (10µl, 10% v/v), cytotoxicity was measured by release of LDH as previously described (Section 3.2.4.2.).

An initial experiment (Fig. 26a) indicated a poosible cytotoxic effect of a particular myasthenic

serum in the presence of complement, cytotoxicity <40% for serum without complement and >50% in the presence of complement. Subsequent experiments using the same serum sample under identical conditions were not able to reproduce this result (Fig. 26b). A different myasthenic serum sample (Fig. 26c) also failed to show an obvious effect of complement-mediated cytotoxicity.

3.2.4.6. Reduced titration range.

From previous experiments, it was noted that complement mediated cytotoxicity was most marked at a low added concentration of serum (1µl, 1% v/v, see "Fig. 25e and f"). This effect was further investigated.

Quadruplicate cultures of TE671 cells were set up as previously described (Section 2.11.2.). The cultures were incubated with fresh or stored heat inactivated myasthenic serum samples (0-4µl, 0-4% v/v) in the presence or absence of GPC (10µl, 10% v/v) for 3h at 37°C, cytotoxicity was calculated as previously described (Section 2.11.4.2.).

Complement-mediated cytotoxicity was more apparent using fresh serum (Fig. 27a) than with stored serum (Fig. 27b), again at the lower added concentrations of serum. However, such an effect was not consistent over the titration range.

Fig. 26. Cytotoxic effect of fresh myasthenic sera and complement on TE671 cells, monitored by release of cytosolic LDH.

TE671 cells in 96 well plates (Section 2.11.2.) were exposed to fresh myasthenic serum (0 -10µl, 0 - 10% v/v) in the presence (■) or absence (□) of GPC (10µl, 10% v/v). Cytotoxicity was calculated as previously described.

a) Single representative experiment using a myasthenic serum sample (MG10). Each point is the mean of quadruplicate culture wells.

b)Pooled results of a myasthenic serum (MG10). Results are the mean ± SEM 5 individual experiments.

c)Pooled results of a myasthenic serum (MG11). Results are the mean ± SEM 4 individual experiments.

FIG. 26 Cytotoxic effect of fresh myasthenic sera and complement on TE671 cells, monitored by release of cytosolic LDH.

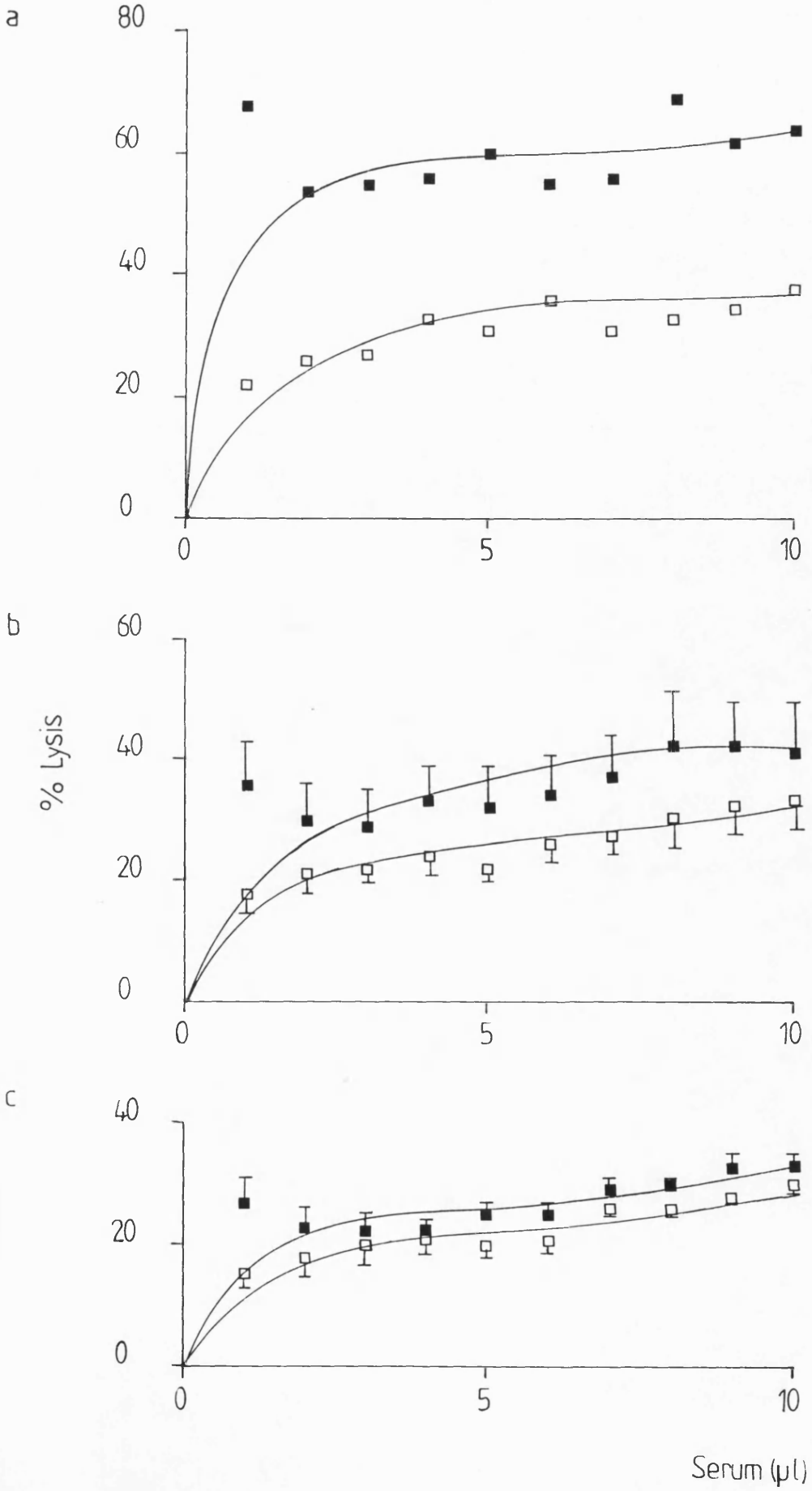


Fig. 27. Cytotoxicity of fresh and stored myasthenic serum and complement on TE671 cells, monitored by the release of cytosolic LDH.

TE671 cells in 96 well plates (Section 2.11.2.) were exposed to fresh and stored heat inactivated myasthenic sera (0 - 4µl, 0 - 4% v/v) in the presence or absence of GPC (10µl, 10% v/v). After incubation (3h, 37°C) cytotoxicity was calculated as previously described (Section 2.11.4.2.).

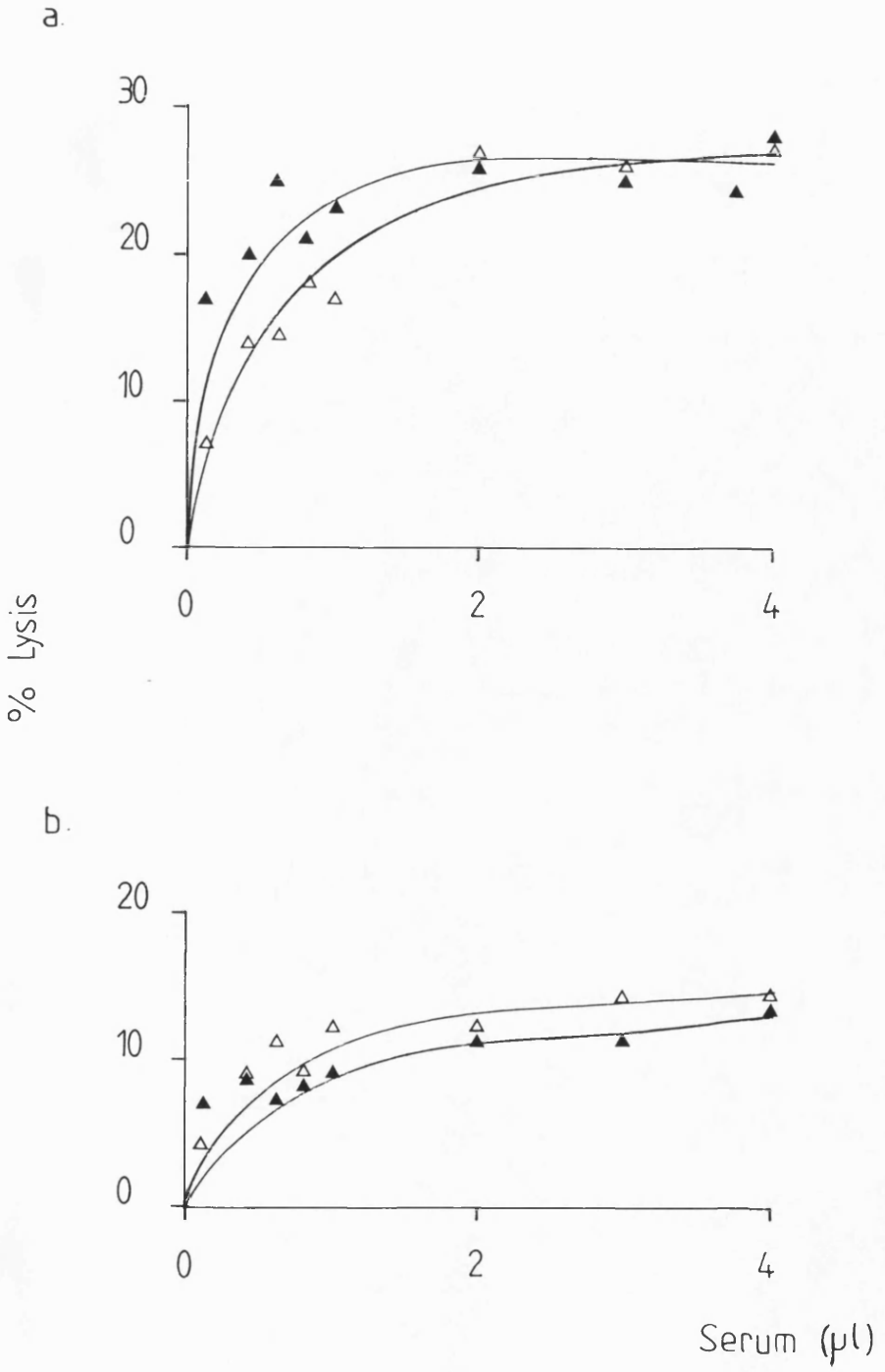
a)Fresh myasthenic serum (MG9) without GPC (△)

fresh myasthenic serum (MG9) with GPC (▲)

b)Stored myasthenic serum (MG3) without GPC (△)

Stored myasthenic serum (MG3) with GPC (▲)

FIG. 27 Cytotoxicity of fresh myasthenic serum and complement on TE671 cells, monitored by the release of cytosolic LDH.



3.2.4.7. Heat inactivation of serum.

Quadruplicate cultures of TE671 cells were set up as previously described (Section 2.11.2.). Two fresh myasthenic samples (0-4µl, 0-4% v/v) which had been heat inactivated immediately before storage or left untreated, were tested for cytotoxicity in the presence or absence of GPC (10µl, 10% v/v) as previously described (Section 2.11.4.2.).

The results show that the abolition of endogenous complement in the serum by heat treatment (Fig. 28b and d) had no effect compared with serum that had not been heat inactivated (Fig. 28a and c).

3.2.5.1. Endogenous LDH in serum: detection.

TE671 cells have high levels of cytosolic LDH, as determined by lysis with Triton X-100 (Section 3.2.4.1.). However, specific complement-mediated lysis by myasthenic serum samples remained elusive. Although endogenous levels of LDH in the serum samples used in the assays were assumed to be too low to affect the cytotoxicity assay, these levels were checked carefully.

Quadruplicate samples of serum (0-10% v/v in HBSS, 100µl) in 96 well plates, were assayed for the presence of LDH, as described for TE671 cells after lysis with Triton X-100 (Section 2.11.4.1.). Apart from purified

Fig. 28. Effect of heat inactivation of myasthenic sera and complement on release of cytosolic LDH.

TE671 cells in 96 well plates (Section 2.11.2.) were exposed to increasing concentrations of two myasthenic serum samples (0 - 4µl, 0 - 4% v/v) in the presence (▲) or absence (△) of GPC (10µl, 10% v/v). After incubation (3h, 37°C), cytotoxicity was calculated as previously described.

a)Myasthenic serum (MG12) non-heat inactivated

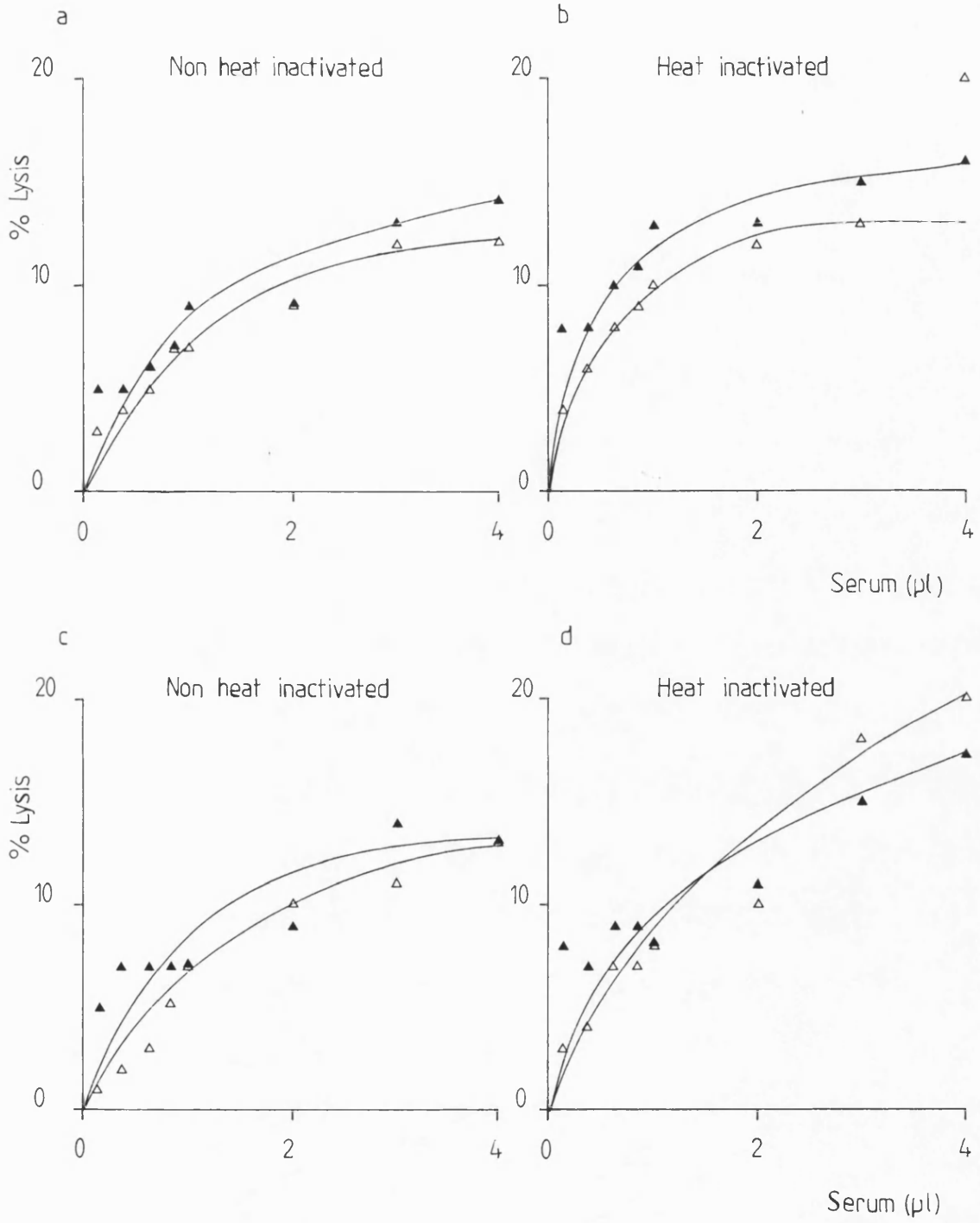
b)Myasthenic serum (MG12) heat inactivated

c)Myasthenic serum (MG13) non-heat inactivated

d)Myasthenic serum (MG13) heat inactivated

Results are from represntative experiments and are the mean of quadruplicate culture wells.

FIG. 28 Effect of heat inactivation of myasthenic sera and complement on release of cytosolic LDH



IgG fractions, all the serum samples showed LDH (Fig. 29ab). However, the levels only represented 20-50% of the LDH that could be released by Triton X-100 (Section 3.2.4.1.).

3.2.5.2. Endogenous LDH isoforms in serum: selective inhibition or removal.

LDH has several isoenzymes, the isoform predominating in blood is LDH1, although myasthenic serum samples may have some LDH5 as a result of damage at the muscle end-plate. The LDH isoenzymes can be differentiated by heat denaturation (Bergmeyer and Bernt, 1974). Serum samples were routinely heat inactivated (56°C, 1h) and further heat treatment was thought to be unnecessary.

3.2.5.3. Inhibition by oxalate.

LDH1 can be selectively inhibited by 0.2mM oxalate. Quadruplicate serum samples (100µl, 0-10% v/v in HBSS containing 0.2mM oxalate, final concentration) were assayed for the presence of LDH as described above. Inclusion of oxalate in the assay buffer had little effect on inhibition of serum LDH levels with respect to serum samples in HBSS without oxalate (Fig. 30abc and d).

3.2.5.4. Separation of isoforms on DEAE-Sephadex A-50.

The individual isoforms of LDH can also be separated from each other by chromatography. The most effective method for separating LDH1 is by adsorption on DEAE-Sephadex.

DEAE-Sephadex A-50 was prepared and mixed with serum samples as previously described (Section 2.11.4.3.). Samples treated with 0.2mM oxalate and subsequently adsorbed onto the matrix were also investigated. Samples of the resulting eluates were assayed for LDH activity as described above. Dilution of the serum after treatment with the matrix was taken into account. The effect of this treatment is shown in Fig. 30 (a,b,c and d). Adsorption resulted in a 50-60% lowering of serum LDH levels in both normal and myasthenic serum samples with respect to untreated samples and those treated with 0.2mM oxalate. There was no apparent advantage afforded by 0.2mM oxalate in the HBSS or with its use in conjunction with DEAE-Sephadex A-50.

3.2.5.5. Removal of LDH on Matrex Blue-gel A.

An additional method considered for the removal of serum LDH was the use of Matrex Blue-gel A. This is a cross-linked 5% agarose covalently coupled to Cibacron Blue 3GA and specifically removes all NAD^+-dependent enzymes.

Fig. 29. Optical density (OD492) of sera used in cytotoxicity assays, measurement of endogenous LDH.

Serum samples in HBSS (100µl, 0 - 10% v/v) were tested for LDH activity as described (Section 2.11.4.1.).

a)Increasing concentrations of:

(□)Pooled normal human serum

(■)GPC

(●)Normal human IgG

(○)Myasthenic IgG (MG1)

b)Increasing concentrations of myasthenic sera:

(■)MG12

(□)MG11

(○)MG13

(△)MG3

(●)MG6

Results are the mean of quadruplicate culture wells.

FIG. 29 Optical density (OD 492nm) of sera used in cytotoxicity assays, measurement of endogenous LDH.

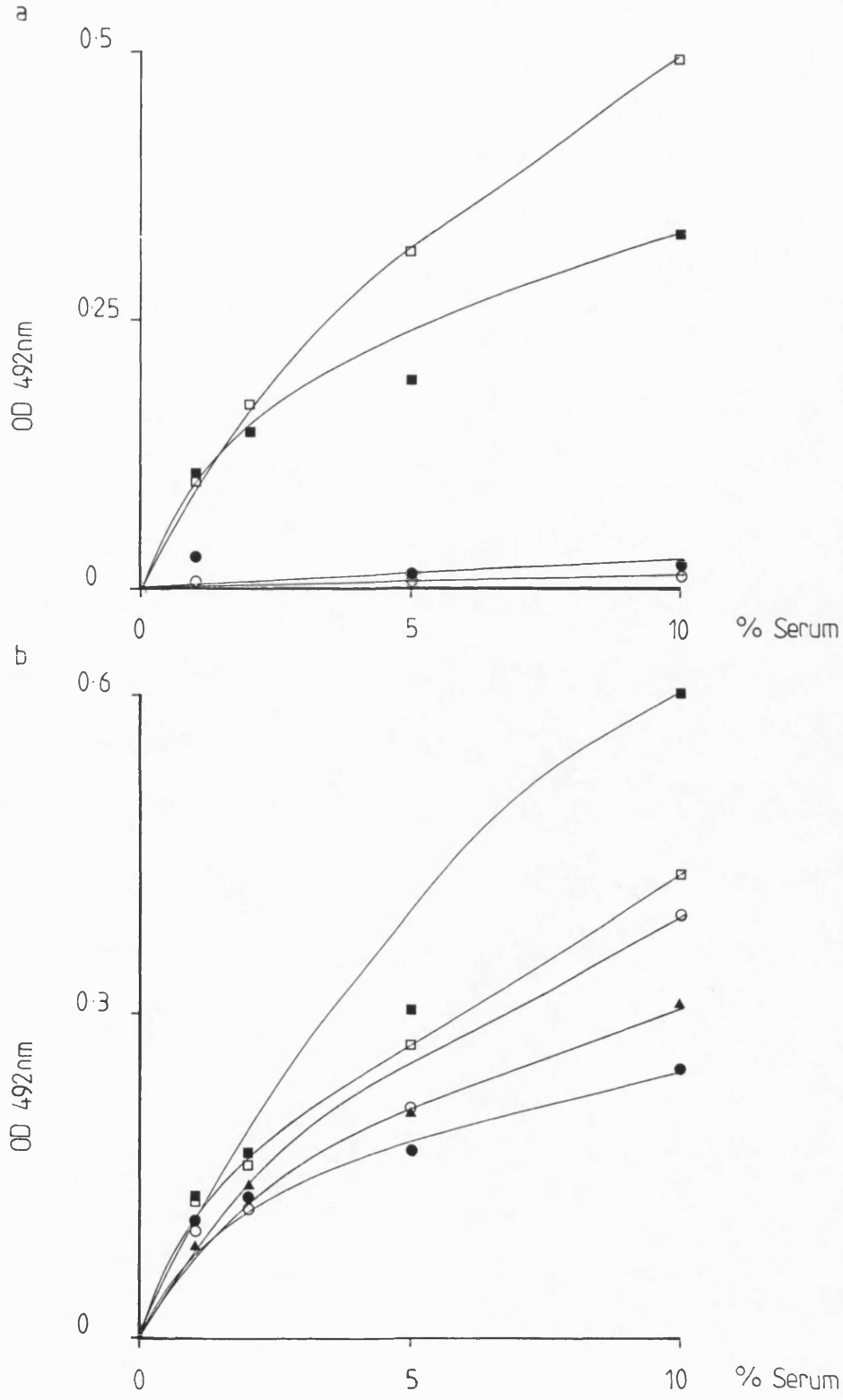


Fig. 30. Optical density (OD492) of sera used in cytotoxicity assays after selective inhibition of LDH.

Serum samples in HBSS (100µl, 0 - 10% v/v) were tested for LDH activity as previously described (Section 2.11.4.1.), after the following treatments:

(●)0.2mM oxalate in HBSS

(■)Adsorption onto Sephadex A-50

(□)Adsorption onto Sephadex A-50 andtreatment with 0.2mM oxalate in HBSS

(○)No treatment.

a)Pooled normal human serum

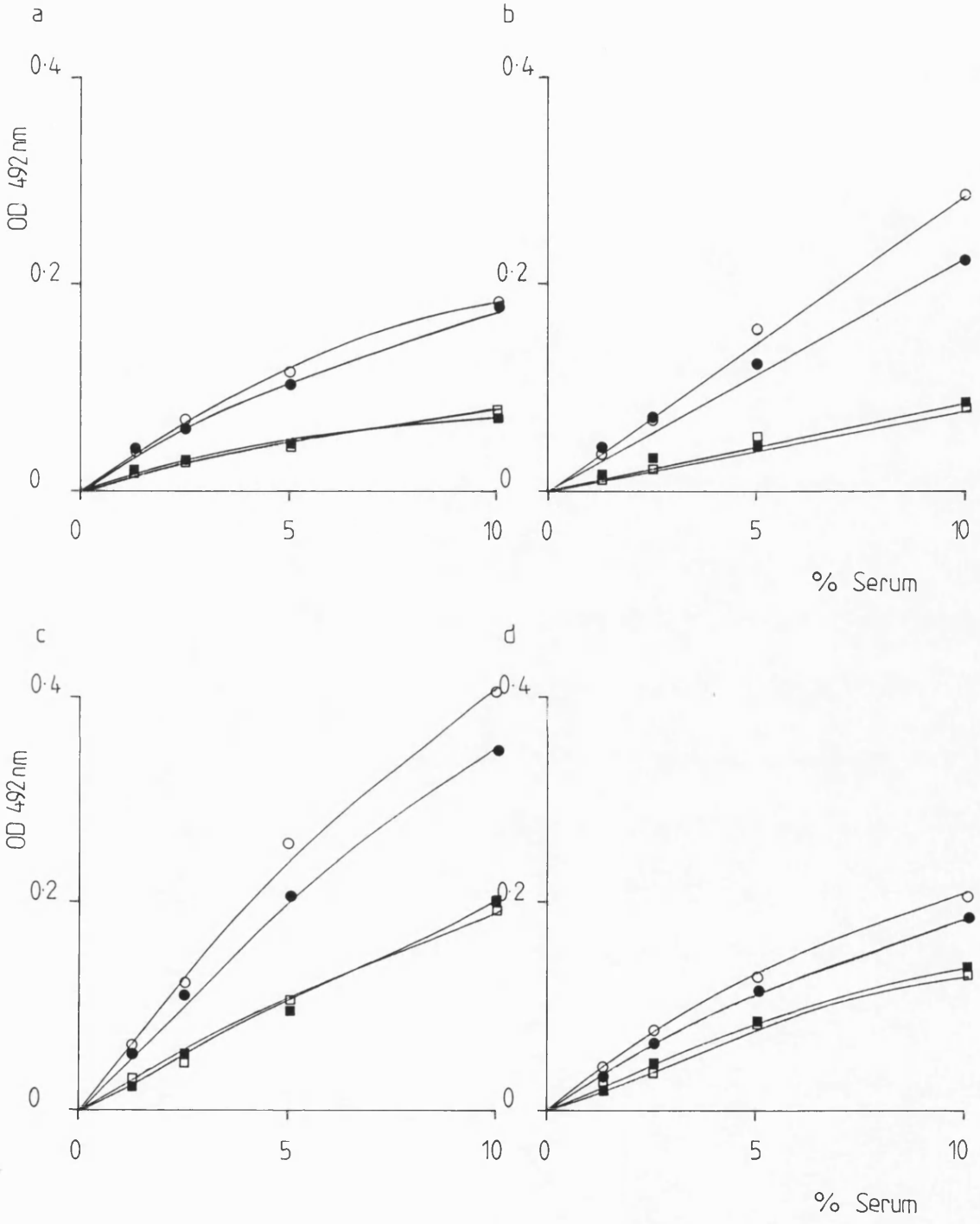
b)Myasthenic serum (MG13)

c)Myasthenic serum (MG12)

d)Myasthenic serum (MG3)

Results are the mean of quadruplicate culture wells tested in duplicate.

FIG. 30 Optical density of sera used in cytotoxicity assays after selective inhibition of LDH.



Matrex Blue-gel A pretreated as described in Section 2.11.4.3.) and used to adsorbe serum LDH as described above (Section 3.2.5.4.). This treatment was no more effective in adsorbing the LDH than that using Sephadex A-50 (Fig. 31).

3.2.5.6. Effects of adsorption on anti-AChR antibody titre.

A potential problem of adsorbing sera onto either of the two matrices, Sephadex A-50 or Matrex Blue-gel A, is the concommitant adsorption of myasthenic immunoglobulins. This would render a serum less useful for investigation of complement mediated lysis.

The standard radioimmunoassay for anti-AChR antibodies (Section 2.6.) was performed on untreated sera and on those adsorbed onto the matrices. The results are summarised in Table 12., where it can be seen that treatment with A-50 resulted in less antibodies being removed than with Blue-Gel.

3.2.5.7. Effect of adsorption on the cytotoxic effects of serum and complement on TE671 cells.

A pretreated myasthenic serum (adsorbed onto either Blue-Gel or Sephadex) was tested for complement-mediated cytotoxicity, measured by the release of LDH as previously described (Section 2.11.4.2.). Using either

treatment, A-50 or Blue-Gel, there was no difference in levels of cytotoxicity in the presence or absence of GPC (Fig. 32a and b).

3.2.6. Radiolabel.

3.2.6.1. Uptake of L-[Me-^{3}H] carnitine and [^{51}Cr] by TE671 cells.

TE671 cell cultures were tested for uptake of radioactivity after incubation with L-[Me^{3}H] carnitine or [^{51}Cr] as described (Section 2.11.5.). Figs. 33 and 34 illustrate that uptake of radiolabel in both cases was not saturating over the concentration range tested (0 - 10µM carnitine; 0 - 0.2µCi chromium). Saturation with chromium was still unattainable after increasing the concentration from 0 - 20µCi.

TE671 cells in culture are not contaminated by additional cell types that would reduce the specificity of a cytotoxicity assay system similar to that found with human myotube cultures (Section 1.11.1.). TE671 cells also represent a continuous supply of material for assay. [^{51}Cr] was chosen as the radioligand to help reduce the cost of the assay.

3.2.6.2. Effect of washing on release of [^{51}Cr] from TE671 cells.

TE671 cells were labelled with [^{51}Cr] (0-20µCi/well) by

Fig. 31. Comparison of Sephadex A-50 and Matrex Blue-gel as methods for adsorption of serum LDH.

A myasthenic serum (MG12) in HBSS (100µl, 0 - 10% v/v) was adsorbed onto Sephadex A-50 (■), Matrex Blue-gel (□) or left untreated (○) and tested for LDH activity as previously described.

Results are the mean of quadruplicate culture wells.

FIG. 31 Comparison of Sephadex A-50 and Matrex Blue-gel as methods for adsorption of serum LDH.

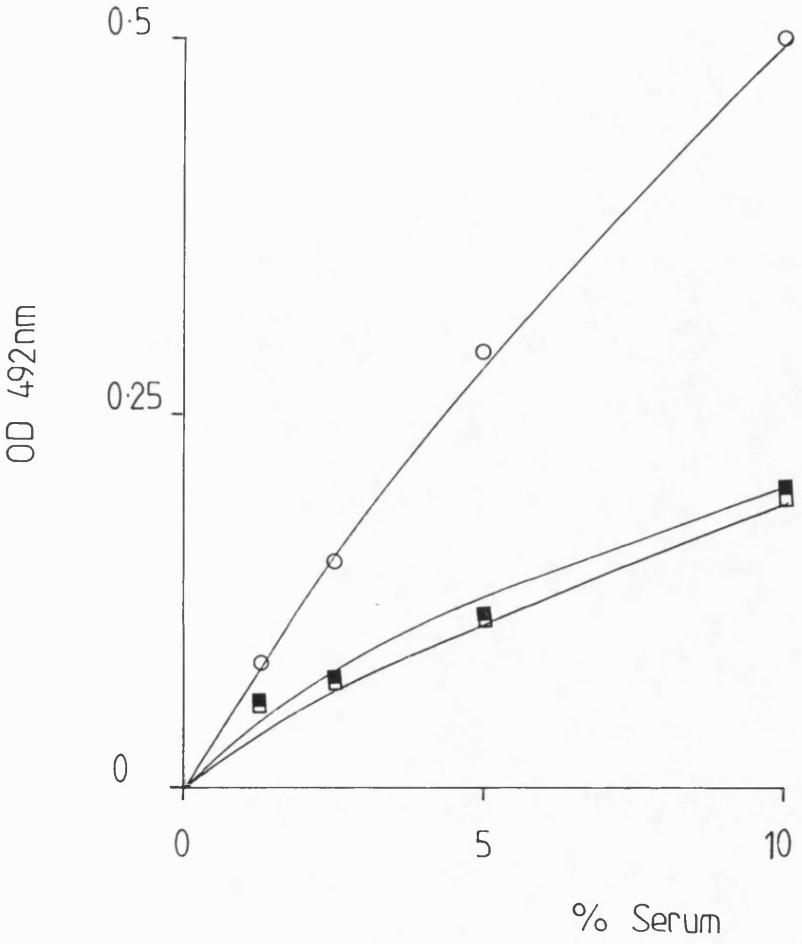


Table 12. Effect of adsorption on anti-AChR antibody titre.

	Anti-AChR antibody titre (nM)				
Serum	Untreated	A-50	% Loss antibody	Blue-gel	% Loss antibody
NHS	-	-	-	-	-
MG13	35.1	31.0	11.7	25.0	29.0
MG12	24.6	21.9	11.0	16.4	33.0
MG3	71.3	69.2	3.0	-	-

Fig. 32. Cytotoxicity of adsorbed serum and complement on TE671 cells, monitored by release of cytosolic LDH.

TE671 cells in 96 well plates (Section 2.11.2.) were exposed to an adsorbed myasthenic serum sample (MG12), (0 - 10µl, 0 - 10% v/v) in the presence (●) or absence (O) of GPC (10µl, 10% v/v).

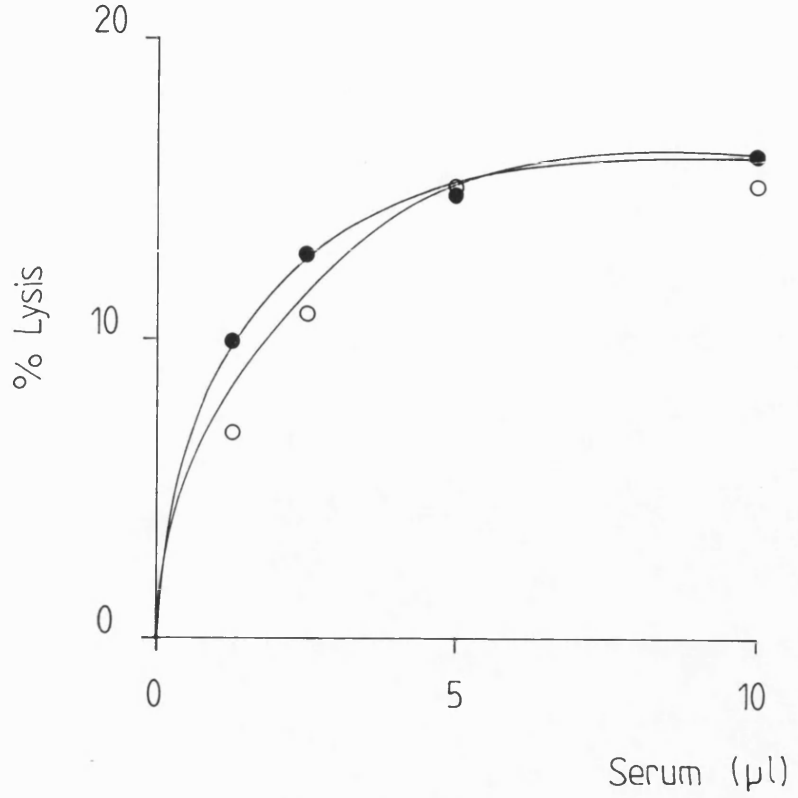
a)Serum adsorbed onto Sephadex A-50

b)Serum adsorbed onto Matrex Blue gel

Cytotoxicity was calculated as previously described. Results are the mean of quadruplicate culture wells tested in duplicate.

FIG. 32 Cytotoxicity of adsorbed serum and complement on TE671 cells, monitored by release of cytosolic LDH.

a



b

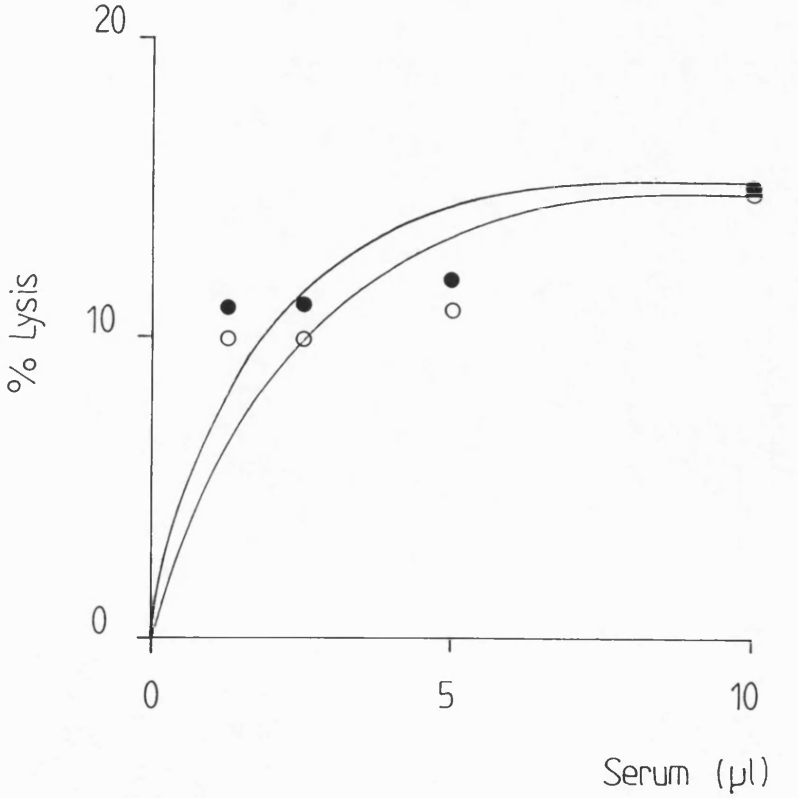


Fig. 33. Uptake of L-[Me-^{3}H] carnitine by TE671 cells.

The uptake of radioactivity by TE671 cells in culture was determined after incubation with increasing concentrations of L-[Me-^{3}H] carnitine for 18h at 37°C as described in Section 2.11.5.. Results are from 1 representative experiment, each concentration is the mean of 4 culture wells.

FIG. 33 Uptake of L-[Me-^{3}H]-carnitine by TE671 cells in culture.

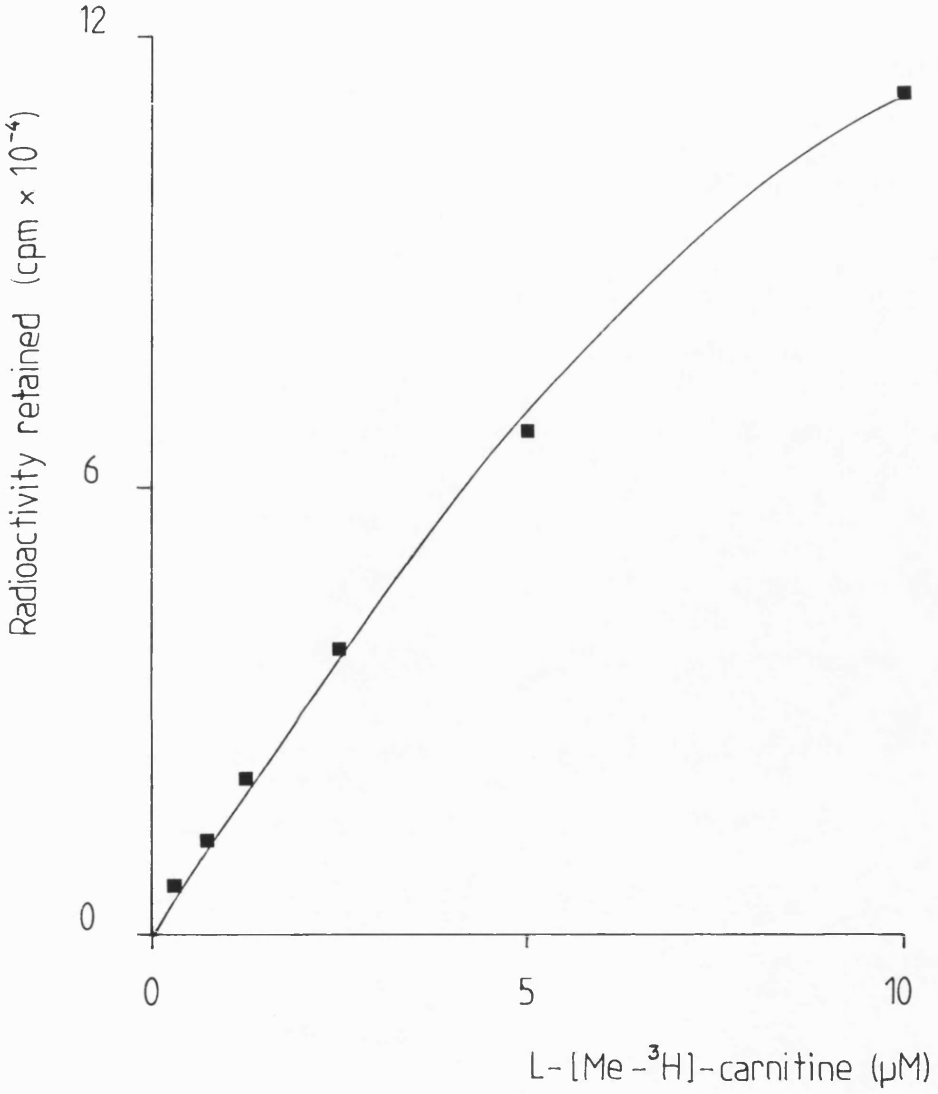
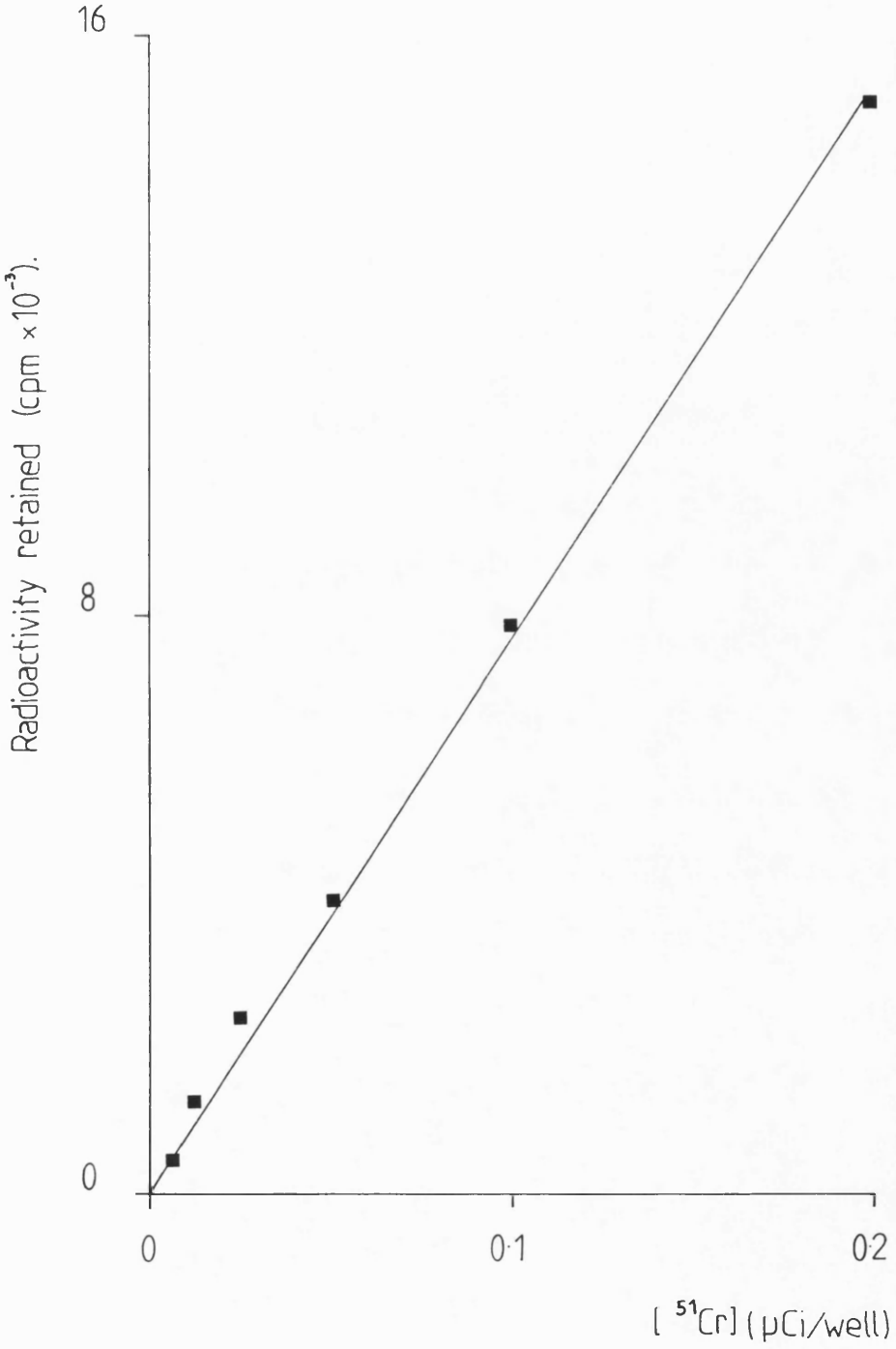


Fig. 34. Uptake of [^{51}Cr] by TE671 cells in culture.

The uptake of radio activity by TE671 cells in culture was determined after incubation with increasing concentrations of [^{51}Cr] for 18h at 37°C as described in Section 2.11.5.. Results are from 1 representative experiment, each concentration is the mean of 4 culture wells.

FIG. 34 Uptake of [^{51}Cr] by TE671 cells in culture.



incubation for 18h at 37°C (Section 2.11.5.). Cells were then washed, fresh medium (0.5ml) was added and the cultures were further incubated (37°C, 3h). After this time, replicate culture wells were washed and their contents were solubilised and counted for radioactivity. Fig. 35 shows the amount of radioactivity lost from the cells to the medium and that retained within the cells before and after washing. Loss of radioactivity was minimal and essentially linear with [^{51}Cr] content release being no higher than 9% in the incubation period (3%/h).

3.2.6.3. Effects of lytic agents on [^{51}Cr] labelled TE671 cells.

After labelling with [^{51}Cr] (2.5μCi/well, 18h, 37°C; Section 2.11.5.). TE671 cell cultures were washed and the effects of Triton X-100 and distilled water were tested. Fig. 36 illustrates that lysis could be effected by concentrations above and including 0.05% (v/v) Triton X-100 and by distilled water alone. Higher added concentrations of Triton X-100 were tested (0.25-1.0% v/v), these concentrations effected detachment of cells from the culture plates.

3.2.6.4. Cytotoxicity of serum on [^{51}Cr] labelled TE671 cells: Titration of serum.

A cytotoxicity assay based on the L-[Me-^{3}H]

Fig. 35. Effect of washing on release of [^{51}Cr] from TE671 cells.

Quadruplicate wells were labelled with increasing concentrations of [^{51}Cr] as described in Section 2.11.5.. The cultures were washed and fresh growth medium containing no radiolabel was added. The retention of radioactivity in the cells after a 3h incubation period at 37°C without washing (□) and after 3 washes (■) was determined. The medium above the cells was removed for counting as an indication of radioactivity released (△) results are from two independent experiments.

FIG. 35 Effect of washing on release of [^{51}Cr] from TE671 cells in culture.

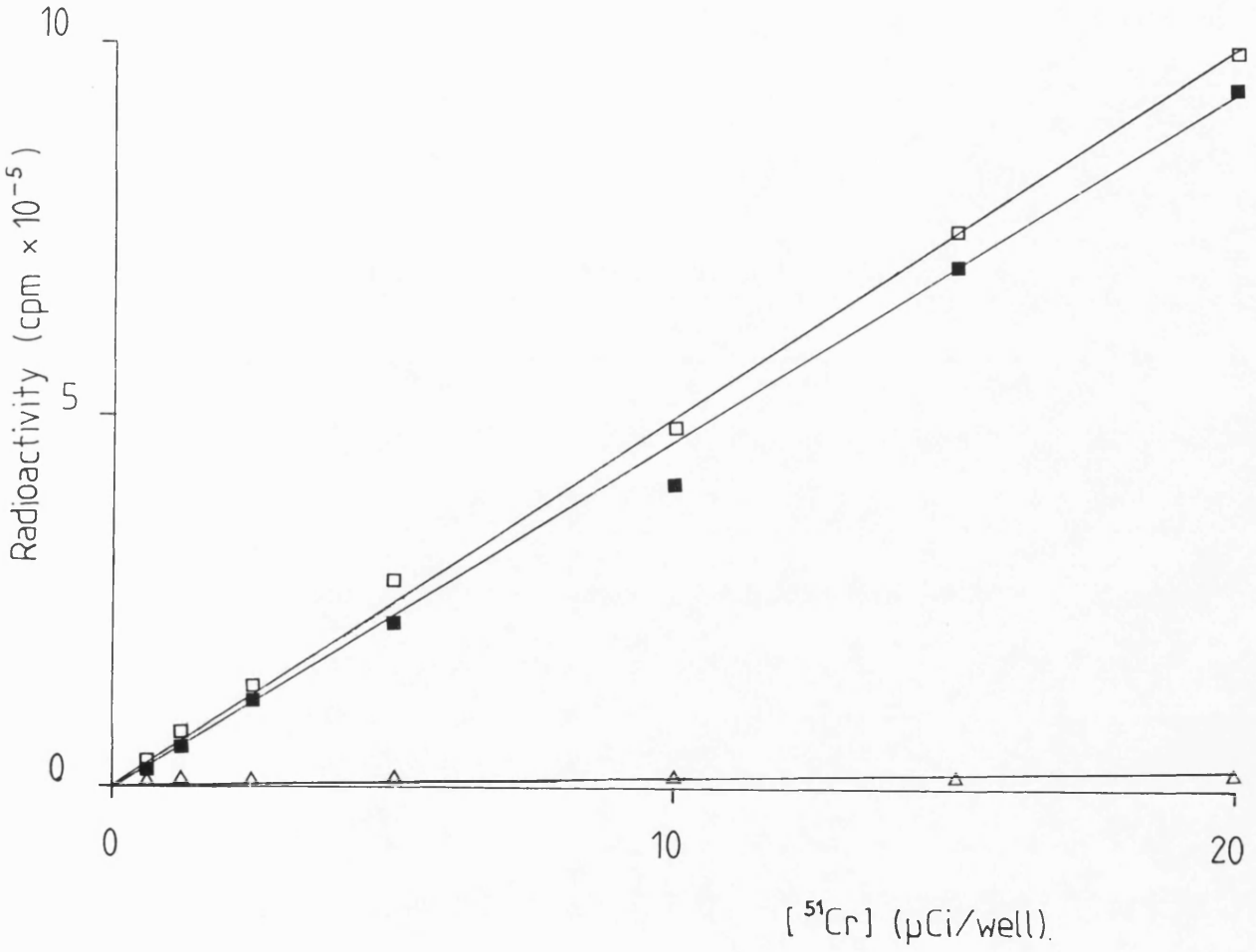
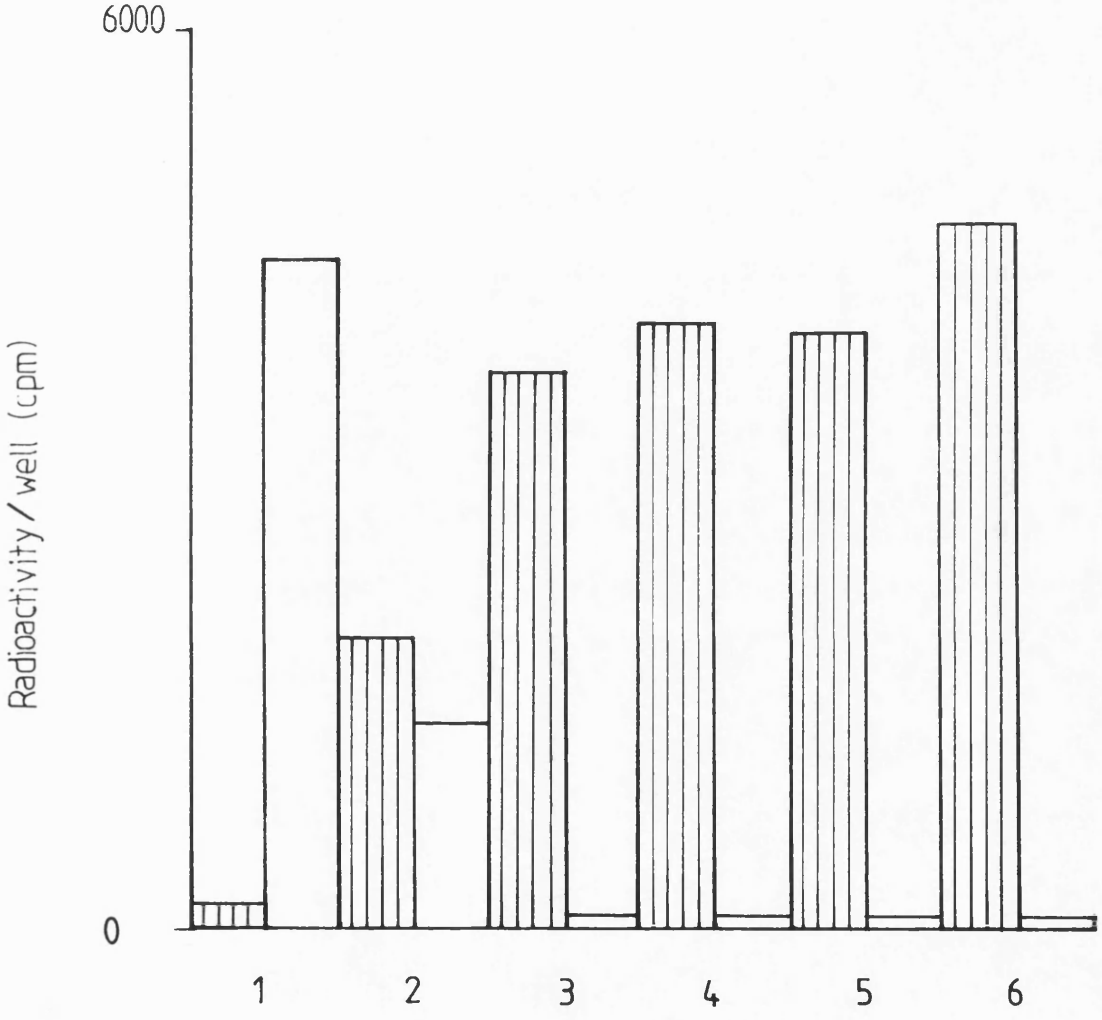


Fig. 36. Effects of lytic agents on [^{51}Cr] labelled TE671 cells.

TE671 cells were labelled with [^{51}Cr] (2.5μCi/well) for 18h at 37°C. Lysis was effected by addition of fresh medium containing Triton X-100 or double distilled water for 30 min, 37°C. Medium above the cells (hatched bars) was removed and counted for radioactivity. The cells remaining were washed, extracted and counted for radioactivity (open bars) as previously described (Section 2.11.5.).

Results are from two resentative experiments.

FIG. 36 Effects of lytic agents on [^{51}Cr] labelled TE671 cells.



carnitine assay monitoring the myotoxicity of serum and GPC on cultured human myotubes (Section 2.10.4.), was used to investigate similar effects on TE671 cells labelled with [^{51}Cr].

The effects of increasing concentrations of heat-inactivated myasthenic serum and a pooled normal human serum (0-100% v/v and 0-90% v/v respectively) were tested on quadruplicate cultures of [^{51}Cr] labelled TE671 cells in 24 well plates. From Fig. 37 it can be seen that cytotoxicity was a function of the amount of serum added. In the case of normal human serum, addition at concentrations greater than 70% (v/v) resulted in detachment of cells from the culture plate.

3.2.6.5. Effect of serum and GPC.

TE671 cell cultures labelled with [^{51}Cr] in 24 well plates as described above, were incubated with increasing concentrations of heat-inactivated serum (0-100% v/v) in the presence of a constant concentration of GPC (150µl). Under these conditions a cytotoxic effect of myasthenic serum and GPC was most marked at 10-20% (v/v) myasthenic serum (Fig. 38). A value of 20% (v/v) serum was chosen for the subsequent cytotoxicity studies.

3.2.6.6. Effect of anti-AChR antiserum.

An antiserum which was raised against the fetal

Fig. 37. Cytotoxicity of serum on [^{51}Cr] labelled TE671 cells.

TE671 cells were labelled with [^{51}Cr] as previously described. Increasing volumes of heat inactivated normal (■) or myasthenic serum samples (□), (0 - 100% v/v) were added and cultures were incubated for 3h at 37°C. Control cultures to which no additions of serum were made were run simultaneously.

At the end of the incubation period, the cultures were washed and extracted for counting. Cytotoxicity was calculated as described in Section 2.11.5.1.. Each addition was tested on 4 culture wells in duplicate.

**
cells detached from culture plate

FIG. 37 Cytotoxicity of serum on [^{51}Cr] labelled TE671 cells.

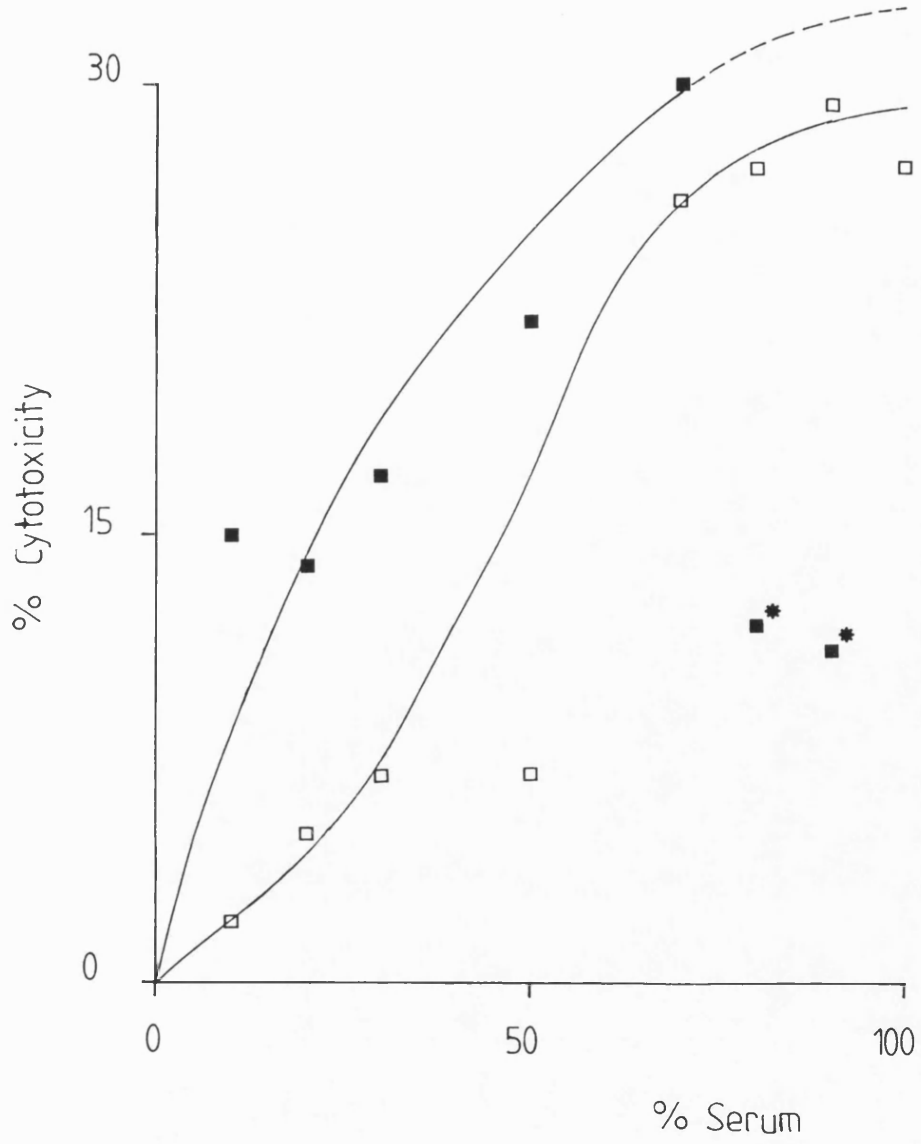
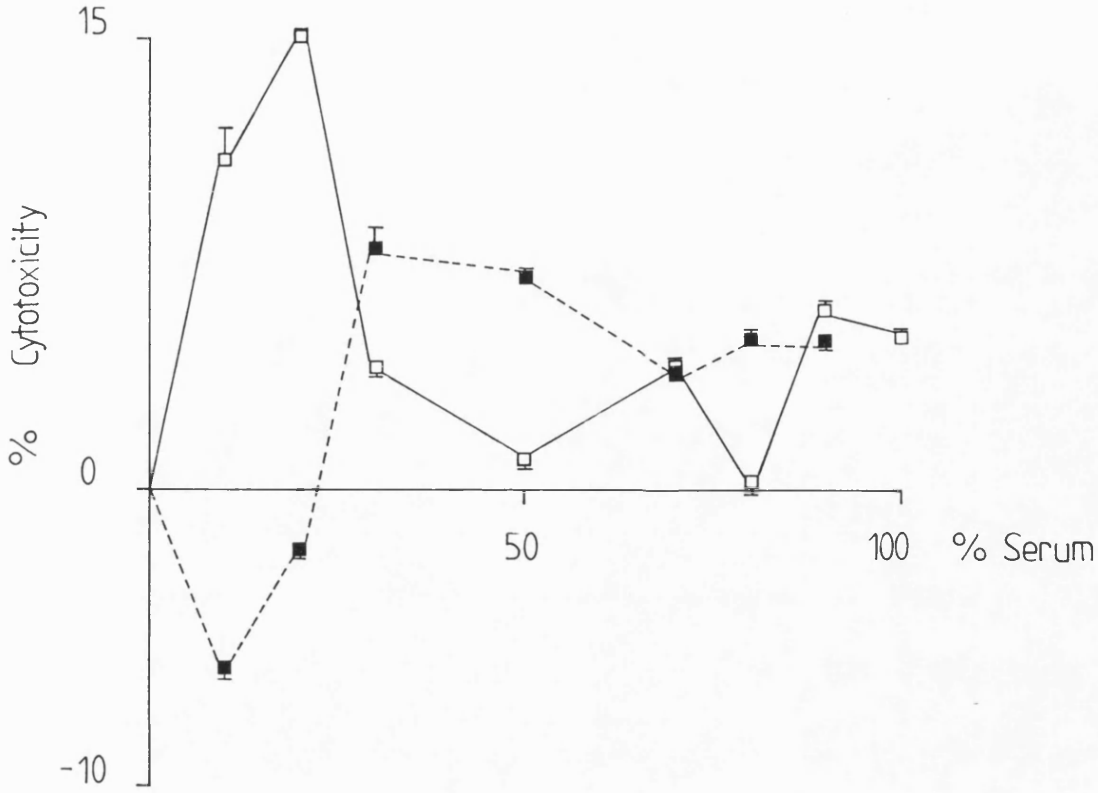


Fig. 38. Cytotoxicity of serum and complement on [^{51}Cr] labelled TE671 cells.

TE671 cells were labelled by incubation with [^{51}Cr] (2.5µCi/well as previously described (Section 2.11.5.). Increasing volumes of heat inactivated normal (■) or myasthenic serum (□), followed by the addition of a fixed volume of GPC (150µl) were added and cultures were incubated for 3h at 37°C. Control cultures to which no additions of serum were made were included in the test.

At the end of the incubation period, the cultures were washed, solubilised and counted for radioactivity. Cytotoxicity was calculated as previously described (Section 2.11.5.1.). Each addition was tested on quadruplicate culture wells. Results are the mean ± SEM of four independent experiments.

FIG. 38 Cytotoxicity of serum and complement on [^{51}Cr] labelled TE671 cells



calf AChR by Ms. S. Walsh of this Department by repeated immunisations with small quantities of purified protein, was tested for complement-mediated cytotoxicity towards TE671 cell cultures by using the experimental conditions previously described (Section 2.11.5.1.). Under these conditions, the antiserum was shown to cross-react with the AChR on TE671 cells (Fig. 39) and the response was comparable to that seen with myasthenic serum (Fig. 38).

3.2.6.7. Cytotoxicity of serum and GPC.

Quadruplicate cultures of TE671 cells labelled with [^{51}Cr] were tested with heat-inactivated serum (20% v/v) in the presence or absence of GPC (as previously described for human muscle cell cultures labelled with L-[Me-^{3}H] carnitine, Section 2.10.4.). Control cultures to which no additions were made (medium alone) were run simultaneously, within each experiment the effect of GPC added alone (150µl) was tested. Cytotoxicity was calculated as previously described.

Under these conditions, the complement-mediated cytotoxicity of serum samples from 6 myasthenic patients and a pooled normal human serum were tested (Table 13 and Fig. 40). Heat-inactivated serum, in the presence or absence of GPC showed wide variation among the treatment regimes, a specific complement-mediated effect of myasthenic serum was not apparent.

Fig. 39. Effect of anti-AChR antiserum on cytotoxicity of TE671 cells labelled with [^{51}Cr].

TE671 cells were labelled with [^{51}Cr] as previously described (Section 2.11.5.). Increasing volumes of anti-AChR antiserum (rabbit anti-(fetal calf AChR) (0 - 50% v/v) in the presence (■) or absence (□) of GPC (150µl) were added and cultures were incubated for 3h at 37°C. Control cultures to which no additions of serum were made were included in the test.

At the end of the incubation period, cultures were washed, solubilised and counted for radioactivity. Cytotoxicity was calculated as previously described.

Each addition was tested on quadruplicate culture wells. Results are the mean of two indepedent experiments.

FIG. 39 Cytotoxicity of anti-AChR antiserum on [^{51}Cr] labelled TE671 cells.

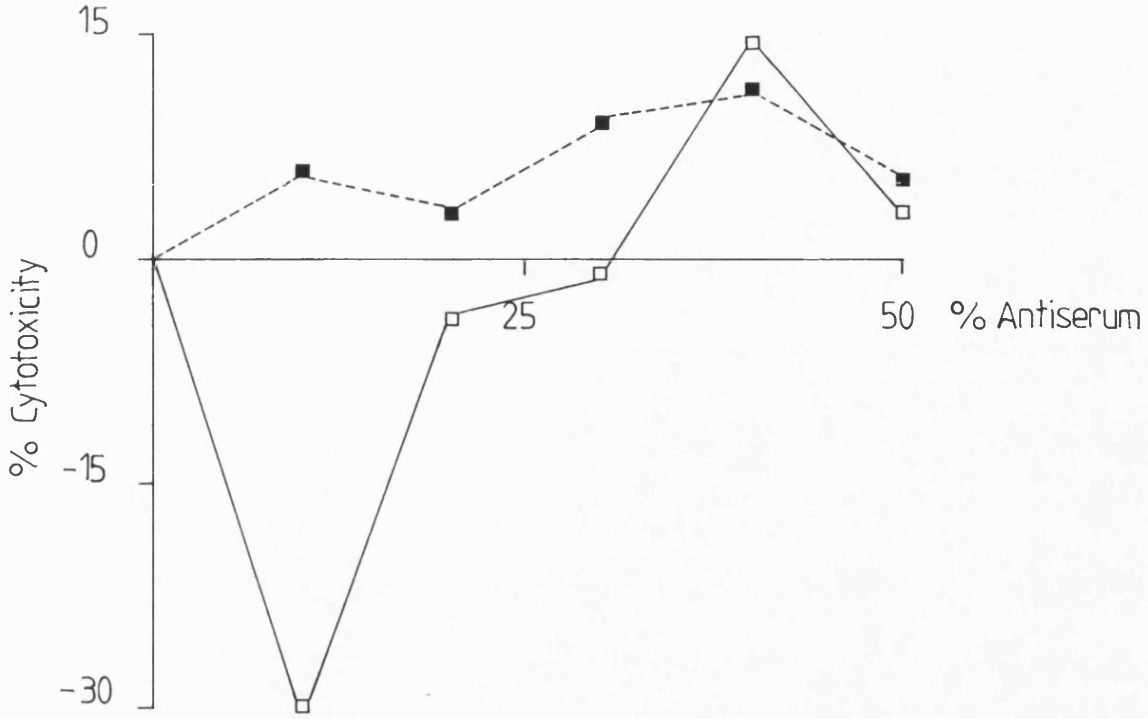


Table 13. Cytotoxicity of serum and complement on [^{51}Cr] labelled TE671 cells.

TE671 cells were labelled by incubation with [^{51}Cr] (2.5µCi/well). The cells were then exposed to samples of myasthenic sera (20% v/v) in the presence or absence of GPC (150µl). Control cultures to which normal human serum was added (in the presence or absence of GPC) and to which GpC alone was added were included in each experiment.

Cytotoxicity was calculated as previously described. Results are the mean of 2 or the mean ± SEM of 4 - 9 independent experiments.

Fig. 40. Cytotoxicity of myasthenic serum and complement on [^{51}Cr] labelled TE671 cells.

Pictorial representation of the data presented in Table 13.

Where: 1 = MG2
2 = MG1
3 = MG6
4 = MG3
5 = MG12
6 = MG14

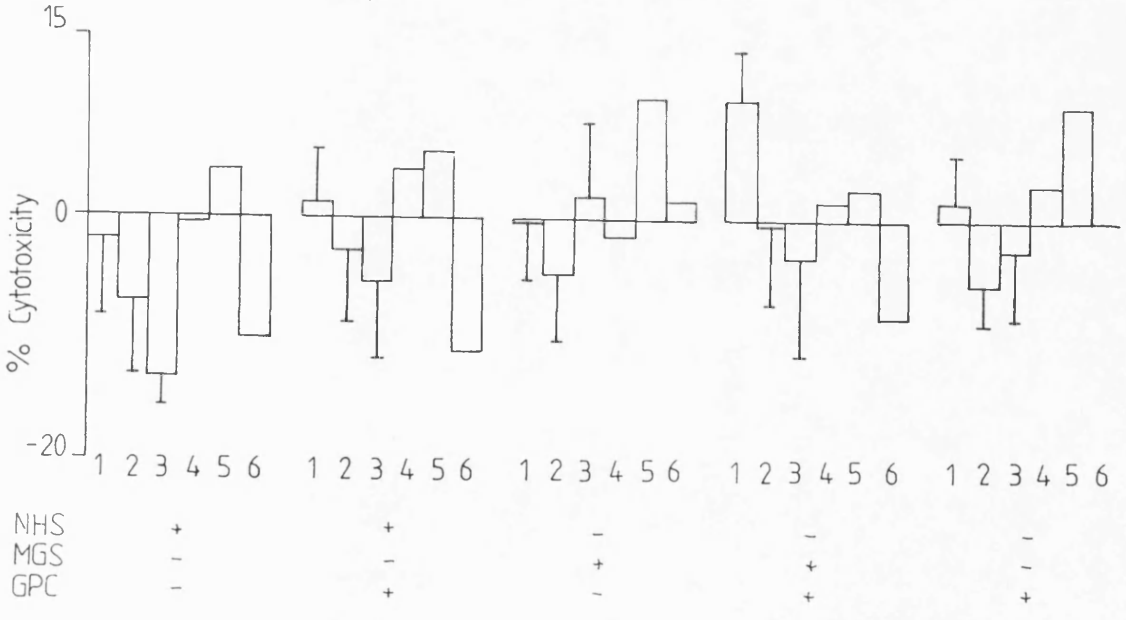
NHS = Normal human serum
MGS = Myasthenia gravis serum
GPC = Guinea pig complement

Table 13. Cytotoxicity of serum and complement on [^{51}Cr] labelled TE671 cells.

Patient	MG2	MG1	MG6	MG3	MG12	MG14
Titre (nM)	20.60	42.00	9.00	71.30	24.60	54.00
Treatment	(n=8)	(n=9)	(n=4)	(n=2)	(n=2)	(n=2)
NHS	-1.75 ±5.81	-7.2 ±6.20	-13.25 ±2.40	-0.5	4.0	-10.0
NHS + GPC	1.13 ±4.60	-2.78 ±5.75	-5.25 ±5.90	4.0	5.50	-11.0
MGS	0.38 ±5.60	4.50 ±5.40	1.75 ±5.90	-1.50	10.0	1.5
MGS + GPC	10.0 ±3.80	-0.67 ±5.70	-3.25 ±7.81	1.50	2.50	-8.0
GPC	1.63 ±3.70	-5.20 ±3.0	-2.50 ±5.40	3.0	9.50	0.0

NHS = Normal human serum
MGS = Myasthenia gravis serum
GPC = Guinea pig complement

FIG. 40 Cytotoxicity of serum and complement on [^{51}Cr] labelled TE671 cells.



3.3.1. C3d degradation products: immunoselection.

The quantitation of complement, particularly C3 and C4, provides useful evidence for the presence of circulating immune complexes. One approach to assess complement consumption is the measurement of C3d, a relatively stable fragment of C3 produced during C3 cleavage. C3d can be separated from the other fragments by its faster electrophoretic mobility or by immunoselection with antisera of well defined specificity (Section 2.9.).

The standard design for C3d quantitations by double-decker rocket immunoelectrophoresis is shown in Fig. 41 (abc and d), where it can be seen that a specific anti-C3c antibody allows the free electrophoretic migration of C3d into a specific anti-C3d containing gel, while precipitating C3, C3b and C3c. In all cases (Fig. 41 abc and d) a standard dilution curve is demonstrated with the C3d preparation (for details, see "Fig. 41 legend"). A dose response curve in the form of smaller, sharply delineated rockets are also seen in the anti-C3c containing gel. These precipitates are formed by breakdown products from C3 with C3b and C3c specificities. Rockets in the same position (in the B gel) are also seen when plasma samples from patients or control plasma are examined. The rocket height tends to be greater for patient plasma samples. In Fig. 41 (b), protein smearing was seen in the myasthenic patient EDTA-plasma samples, subsequent assays (Fig. 41 c and

Fig. 41. C3 degradation products: immunoselection.

The design of the standard double-decker immunoelectrophoresis (DD RIE) for the quantitation of C3d was prepared as previously described (Section 2.9.). Gels A and C are native (antibody free) gel, gel B contains anti-C3c antibody and gel D contains anti-C3d antibody.

a)Wells 1 - 7 received dilutions of a standard C3d preparation.

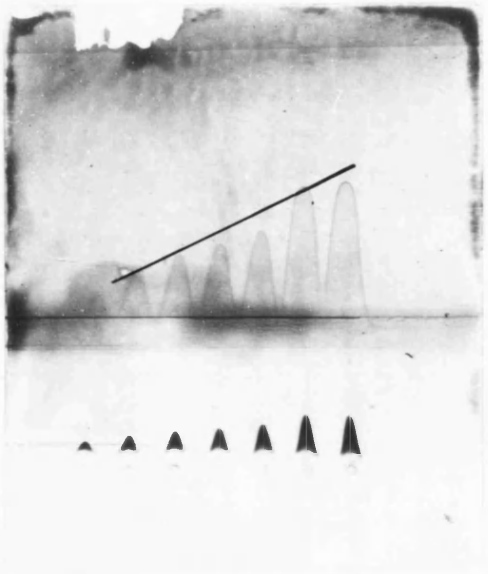
b) Wells 6 - 11 received dilutions of a standard C3d preparation.

Wells 1 - 5 received EDTA plasma from MG patients.

Well.	Patient.
1	10
2	18
3	3
4	16
5	2

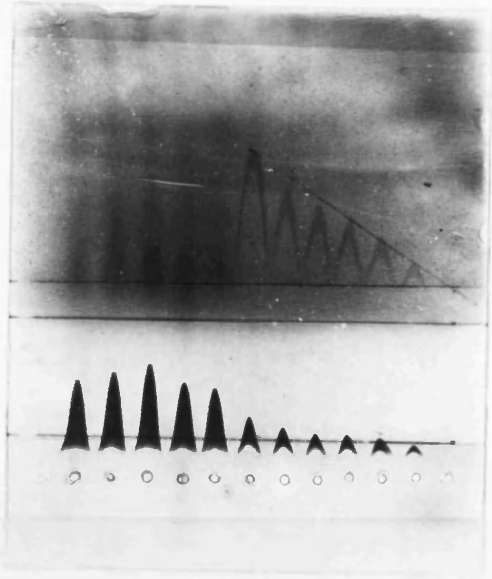
FIG. **41** C3 Degradation products : Immunoselection

a



well 1 7

b



well 1 11

c)Wells 8 - 13 received dilutions of a standard C3d preparation.

Wells 1 - 7 received EDTA plasma from patients with MG.

Well.	Patient.
1	17
2	10
3	18
4	3
5	16
6	15
7	2

d) Wells 16 - 20 received dilutions of a standard C3d preparation.

Wells 9 - 15 received EDTA plasma from patients with MG (as in "c", but having been stored for 6 months, -80°C)

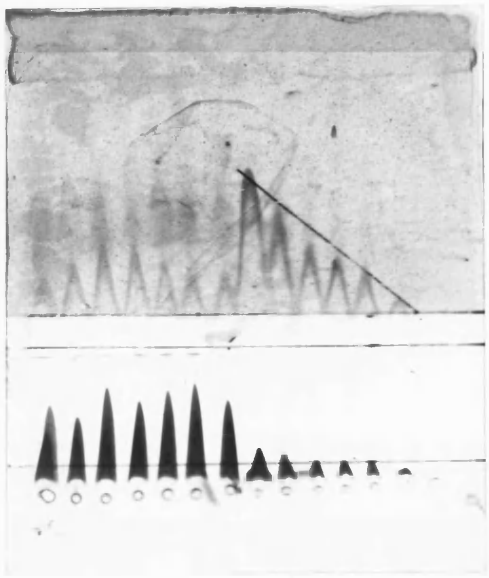
Well.	Patient.
9	MG17
10	MG10
11	MG2
12	MG18
13	MG16
14	MG15
15	MG3

Wells 7 and 8 received EDTA p;asma from two patients with classical rheumatoid arthritis.

Well 6 received EDTA negative control.

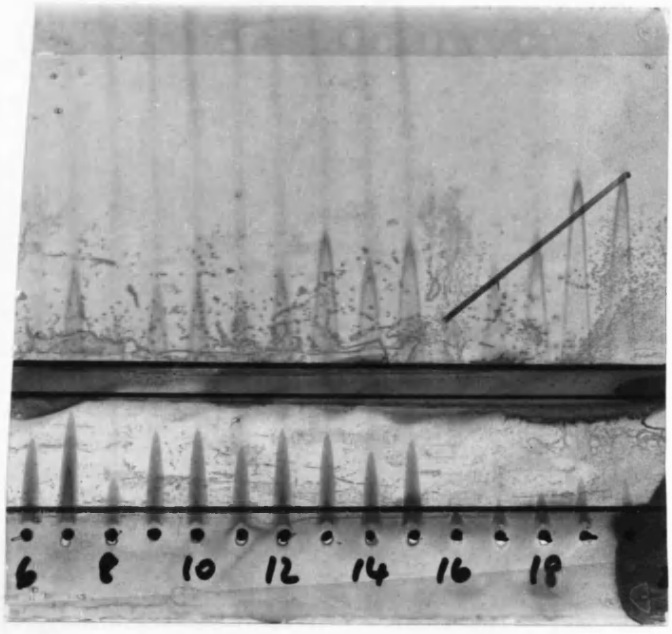
Agarose in Fig. "c and d" contained additionally 1% w/v PEG 6000.

c



well 1 13

d



well

d), employed 1% (w/v) PEG in the agarose gel to help reduce this and to improve the delineation of the protein peaks. When the heights of the C3d peaks are examined, they reveal elevated levels of C3d in all myasthenic patients tested as compared with negative EDTA control plasma and compared with the totally converted C3d standards.

DISCUSSION.

The basic defect in MG is a reduction of AChRs at the neuromuscular junction and it is well established that the neuromuscular abnormalities are brought about by antibody-mediated processes. A great deal has been learned about the autoantibodies against AChRs and the pathogenetic mechanisms by which they produce their effects at the neuromuscular junction in MG (for reviews, see Vincent, 1980; Harrison and Behan, 1986). However, the relationship between circulating antibodies to AChR and the severity of the disease process in myasthenic patients is highly complex. The autoantibodies are heterogeneous and differ in their ability to induce the clinical and pathophysiological features of MG (Drachman et al., 1987). The poor correlation of the absolute serum antibody level with the clinical severity in MG may in part be explained by:

i) A proportion of the antibodies that are bound to AChRs at neuromuscular junctions, and thus do not enter the serum antibody pool.

ii) The serum antibodies may not be accurately measured due to limitations of the radioimmunoassay.

iii) Variations in the concentrations of the IgG subclasses of antibody to AChR.

Determination of anti-AChR IgG subclasses is also relevant to the mechanism of cell destruction in post

synaptic folds. Different IgG subclasses are involved in complement activation and in antibody-dependent cellular cytotoxicity (for review, see Natvig and Kunkel, 1973) which may play a role in MG.

4.1. Mechanisms for antibody loss.

Antigenic modulation is considered one of the two main mechanisms of AChR loss in MG. Antigenic modulation of the AChR has been analysed in tissue culture; where the potency of animal and human antisera on the AChR of animal tissue cultures was investigated (Heinemann et al., 1977; Appel et al., 1979; Conti-Tronconi et al., 1981; Drachman et al., 1982; Hudgson et al., 1982; Tzartos et al., 1985). AChR antibodies in these sera were shown to cause a two- to three-fold enhancement of AChR internalisation. Attempts to correlate severity of disease and ability to cause antigenic modulation produced conflicting results (Conti-Tronconi et al., 1981; Drachman et al., 1982; Hudgson et al., 1982). As the sera were tested on animal cells, only those antibodies able to cross react with the two AChR types would be effective. This was proposed as a serious limitation of the technique, weakening the validity of the results. For further investigation it was suggested that human myotubes with surface AChR were required (Lennon et al., 1983).

There are relatively few reports of human fetal muscle cells in culture, reflecting the difficulty in

supply. Most studies are based on the methods described by Yasin et al. (1977) for the growth of dissociated adult human tissue.

However, Tzartos et al. (1986) realised the potential of the use of human tissue and used human muscle cell cultures to investigate antigenic modulation. The results showed that myasthenic sera could cause AChR loss from such cultures, but there was no correlation with clinical state or severity of the disease (Tzartos et al., 1986). They further suggested that these cultures could be used to investigate other effects of anti-AChR antibodies in MG, like the effect of complement.

4.2. Human muscle cultures for investigation of complement-mediated lysis.

In the present work, cultures of human muscle cells were established. Mononucleated cells settled on the collagen substratum and proliferated; the onset of fusion occuring spontaneously after 3-4 days in culture, aided by the reduction of serum supplement in the medium from 20-10%. The resulting myotubes continued to grow, forming a monolayer of slim cells with branching extensions, interspersed with fibroblasts. Spontaneous contraction of the myotubes was observed in only one of the prepared cultures.

In order to quantify the myolytic effects of myasthenic serum and complement, use was made of a

quantitative assay for muscle cell lysis, originally developed by Cambridge and Stern (1981) to study myotoxicity in polymyositis. The method depends on the preferential uptake of L-[Me-^{3}H] carnitine by cultured myotubes, loss of which can be monitored following cytolytic damage. A major advantage of this procedure was reported to be the very much slower uptake of carnitine by fibroblasts, which as mentioned earlier (Section 1.11.1.), contaminate myotube cultures (Cambridge and Stern, 1981).

In preliminary experiments, concentrations of myasthenic serum and GPC were used which had previously been shown to be myolytic to rat muscle cells in culture (Childs et al., 1984, 1985, 1987). However, when such conditions were applied to human muscle cells in culture, no significant lysis was obtained. It was found to be necessary to use at least a three-fold increase in concentrations of both myasthenic serum and GPC in order to demonstate lysis of the human muscle cells. Thus, Fig. 16 shows the results of increasing both myasthenic serum and GPC on myotoxicity, whereas increasing normal human serum and GPC had little effect.

The modified conditions were subsequently used to investigate a complement-mediated effect of five myasthenic serum samples. The results show that a combination of myasthenic serum and GPC gave higher levels of myotoxicity than the constituent components alone, or than normal human serum in the presence or absence of GPC (Fig. 17). It is not yet clear why such

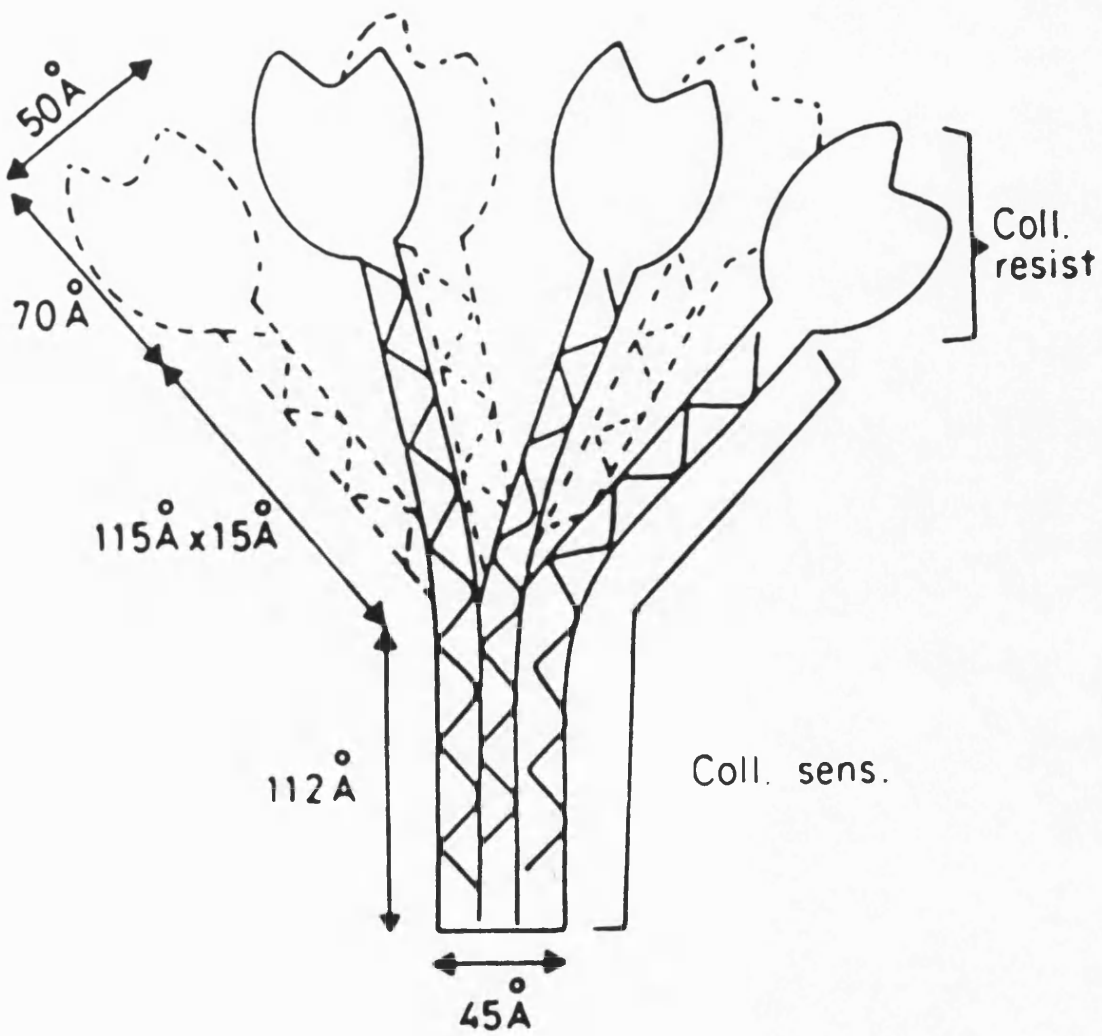
relatively high levels of both serum and GPC are needed to promote lysis in the human muscle cultures. The myotubes may be immature in culture, expressing different antigenic sites, such that sub-populations of antibodies, binding to the cultures, are less so effective at activating GPC. Adams and Bevan (1983,1985) showed that there is a sparse distribution of AChRs on human fetal muscle membranes, at a density of 1-4 receptors per μm, which is considerably lower than the density on other preparations (Table 14).

If classical activation of complement is assumed to be involved in myolysis, C1q, the initial component of the complement cascade, must have two of its six binding sites (Fig. 42) bound to the antigen-antibody complexes to become stable. When IgG is bound to antigen, C1q binds to two separate IgG molecules via their Fc regions. However, the IgG molecules must lie within 30-40nm of each other as this is the maximum distance that C1q can span (Hughes-Jones et al., 1984; Burton, 1985). Dower and Segal (1981) showed that, in C1q binding to antibody coated cells, where antibody is bound to a membrane mobile antigen, C1q will bind preferentially to the region of highest antibody concentration. However, free IgG is a potent inhibitor of C1q binding when cell surface density of bound antibody is low. Therefore, free IgG might modulate C1q binding, preventing C1q fixation and activation on cells bearing low levels of antibody. This lends itself to a possible explanation for the difference in the myotoxic effects of myasthenic

Table 14. Density of AChR on cultured muscle cells.

Muscle source	Receptor density sites/μm^2	Reference
Chick (cluster) embryo (diffuse)	9000 900	Sytowski et al. (1973)
Rat (cluster) embryo (diffuse)	8000 2000	Axelrod et al. (1976)
(cluster) (diffuse)	3-4000 54-900	Land et al. (1977)
Rat L-6 cell line	5-400	Land et al. (1977)
Human fetal	1-4	Adams and Bevan (1983, 1985)

FIG. 42 C1q molecule



serum and GPC on rat and human muscle culture systems, with respect to the number and distribution of the surface antigen (AChR) available (Table 14).

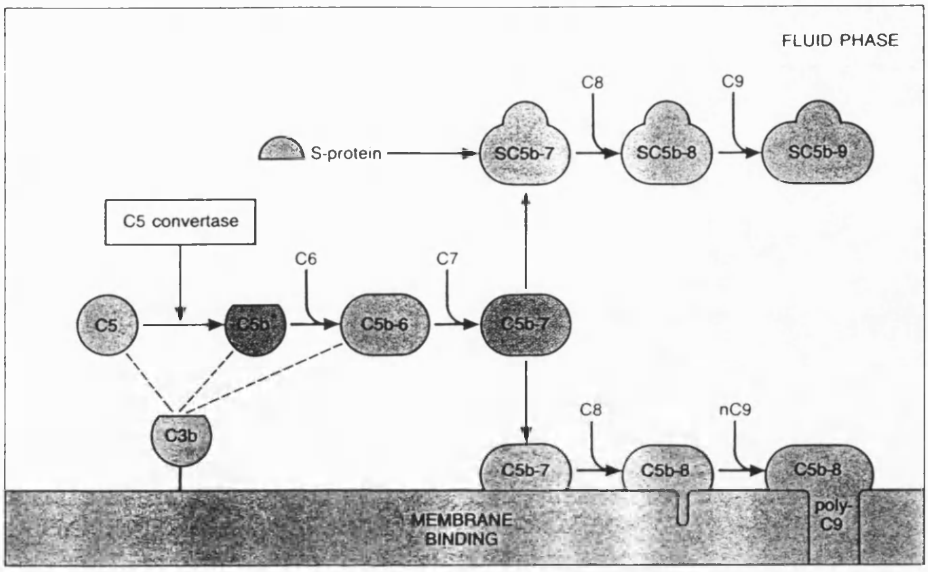
However, once the antibody is bound to the antigen, the lytic phase of the complement pathway is initiated, culminating in the formation of the membrane attack complex (MAC). The assembly of the MAC may be divided into two stages with regard to function, structure and control (Podack, 1986).

1) The insertional event carried out by the trimolecular C5b-7 complex. This reaction proceeds without overt lysis but sets the stage for subsequent membrane damage (Fig. 43a).

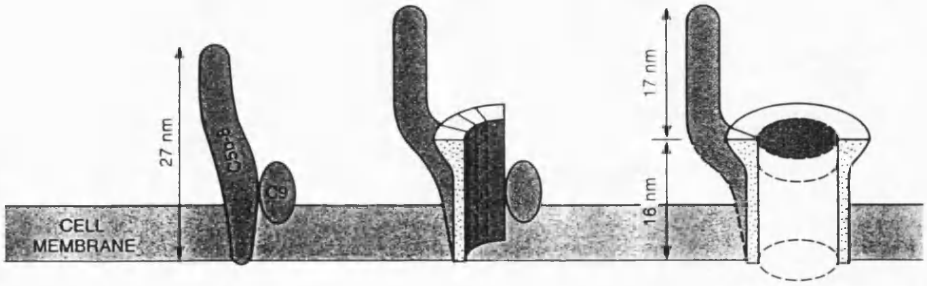
2) Membrane damage by binding of C8 and the polymerisation of C9 to the tubular complex that is responsible for the ultrastructural membrane lesion (Fig. 43b).

The formation of such a lesion in the membrane could cause an uncontrolled influx of extracellular ions, focal calcium excess (Campbell et al., 1979), protease activation and disruption of the cytoskeletal elements within the post synaptic folds. Damaged segments bearing AChR, bound antibody and MAC may be shed into the synaptic cleft. The demonstration by Engel and Arahata (1987) of the MAC at all end plates in 7 cases of MG, reconfirms the effect of complement-mediated injury of the post synaptic membrane in MG. Resealing of the membrane over the folds would then result in a "shallow" post synaptic membrane with fewer

FIG. 43



a). Scheme of assembly of the MAC and its control by S-protein. Metastable forms of C5b and C5b-7 are shaded tan. C5b is loosely bound to membrane C3b. Initial attachment of the assembling MAC is via a transient binding site in C5b-7.



b). A model of the MAC, in which C5b-8 directs polymerization of poly C9 channels which traverse the cell membrane

Taken from Law and Reid (1988)

folds and AChRs than the pre-existing one (Fig. 6).

It was found possible to demonstrate a complement-mediated lytic effect in the human muscle culture system using a limited number of myasthenic sera. However, more rigorous investigation using this system was made difficult by the sporadic availability of tissue of a suitable age and quantity and by the susceptibility of the cultures once established, to infection.

In order to further the investigation of complement-mediated lysis, an alternative and more consistent source of human tissue expressing AChR was sought.

4.3. TE671 human cell line.

The human medulloblastoma cell line, TE671, was chosen, as it had been reported to express an AChR that exhibits electrophysiological, immunological and pharmacological similarities to muscle. Other similarities to muscle, for example, the presence of muscle-associated enzymes were lacking until Stratton *et al*. (1989) noticed that TE671 cells had several of the phenotypic characteristics of striated muscle cells, including the presence of desmin and myoglobin. The amino acid sequences coded by the cDNA for both α and δ subunits of AChRs from TE671 cells show extensive homology with α and δ subunits of AChRs from muscle (Schoepfer *et al*., 1988), unequivocally demonstrating that AChRs from TE671 cells are of the "muscle-type".

During the final stages of this project, it emerged, from DNA fingerprinting studies (Stratton et al., 1989; Stratton, personal communication), that the TE671 cell genotype was identical to that of a human rhabdomyosarcoma cell line isolated from the same laboratory as TE671 (McAllister et al., 1969, 1977). Thus, the TE671 cell line may not be a medulloblastoma of neuronal origin, as originally thought, but does, in the light of this recent evidence, represent an even more suitable homogeneous population of human derived material for the study of human muscle AChRs.

Cultures of TE671 cells were successfully established. The cells in culture were anchorage independent, although a chemical substratum of polylysine was used in routine experimentation to help prevent cell detachment during the washing procedures. Once settled in culture, TE671 cells are polymorphic (Syapin et al., 1982; Zeltzer et al., 1984; Lukas, 1986a): all cell types appear during all stages of culture growth. As the cells reach confluence, those cells with longer cellular extensions were blunted or lost in the massing efect. However, cells are not contact inhibited, continuing to grow over an already established monolayer.

The specific binding of [^{125}I] α-BGT was demonstrated by the use of d-tubocurarine chloride, a cholinergic antagonist. Syapin et al.(1982) had previously demonstrated unusual potency of this drug in inhibiting [^{125}I] α-BGT binding in these cells.

Equilibrium binding studies, using [^{125}I] α-BGT, indicated a high affinity binding site with a Kd of 2.04nM, in agreement with the results of other workers (Table 15). Binding, with respect to the Bmax, was unaffected for up to three days in culture, in agreement with Lukas (1986a), who noted that once the cells achieve over confluence, the density of toxin binding sites per milligram of cellular protein decreases.

Approximately 50% of TE671 cell [^{125}I] α-BGT binding sites were found to be intracellular. There was no significant difference (students t-test) in the results when either α-BGT or d-tubocurarine were used to determine non-specific binding. Intracellular AChRs in muscle cells have been described. Fambrough and Devreotes (1978) reported 20-25% of AChRs on chick myotubes to be intracellular. Approximately half of these receptors were found to be associated with the Golgi apparatus and were inserted into the membrane in the presence of inhibitors of protein synthesis. They also found (Fambrough and Devreotes, 1978), that the remaining 50% of the intracellular receptors were associated with a "hidden" pool, that was not transported to the cell surface, and were degraded at the same rate as cell surface AChRs. Patrick et al. (1977) had similar findings with the BC3H-1 cell line, as did Clementi et al. (1986) with the IMR32 human cell line. Kemp and Edge (1987) however, found that, of a similar intracellular pool of AChRs in PC12 cells, after treatment with cycloheximide, a small proportion were

Table 15. Toxin binding to TE671 cells.

Sample	Kd (nM)	Bmax (fmol/mg protein)	Reference
Intact cells	1.4	44	Syapin et al (1982)
Cell memb.	1.4	22	"
Cell memb.	2.0 60.0	60	Lukas (1986ab)
Intact cells	2.0	24	This study

the immediate precursors of cell surface sites. Carlin et al. (1986) found an intracellular precursor of mature AChR in rat muscle cultures, by immunoprecipitation with monoclonal antibodies directed against the α subunit. This precursor bound α-BGT but was insensitive to the cholinergic ligands d-tubocurarine and decamethonium. Walker et al. (1988) had a similar finding with AChRs found in TE671 cell extracts. This was not found in this study but may reflect different growth stages of the TE671 cells with respect to the internal AChR population.

The role of such intracellular pools of cell surface proteins is not well understood. Intracellular pools of sodium channel α subunits have been identified in developing rat brain (Schmidt et al., 1985) and it has been suggested that the intracellular compartments may be involved in modulating rapid changes in cell surface receptor density.

The pharmacology of [^{125}I] α-BGT binding to TE671 cells is consistant with a "muscle-type" of AChR. The most potent ligands inhibiting [^{125}I] α-BGT binding are the classical AChR antagonists α-BGT, d-tubocurarine and decamethonium; muscarinic and ganglionic AChR ligands showed little effect (Figs. 20 and 21). These results compare favorably with those of other workers (Table 16).

Displacement curves were analysed by Hill plots, where specific binding of radioligand in the absence of any displacing drug is taken as the Bmax and the binding

Table 16. Inhibition of [^{125}I] α-BGT binding to TE671 cells by cholinergic drugs.

	Ki (M)		
Ligand	Syapin et al. (1982) (approx values)	Lukas (1986a,b)	This study
α-BGT	>1.0×10^{-8}	-	6.5×10^{-10}
d-TC	5.0×10^{-7}	2.0×10^{-6}	9.8×10^{-8}
Acetylcholine	-	-	1.7×10^{-7}
Carbamylcholine	2.0×10^{-5}	2.0×10^{-6}	8.7×10^{-7}
Decamethonium	3.0×10^{-5}	5.0×10^{-7}	1.4×10^{-7}
Benzoquinoniuim	-	-	1.25×10^{-6}
(-)-Nicotine	9.0×10^{-6}	1.0×10^{-6}	1.3×10^{-6}
(+)-Nicotine	-	-	2.4×10^{-6}
MLA	-	-	5.2×10^{-5}
Atropine	>3.0×10^{-4}	>3.0×10^{-4}	>1.0×10^{-3}
Hexamethonium	>3.0×10^{-4}	>3.0×10^{-4}	>1.0×10^{-3}
Choline	-	-	>1.0×10^{-3}
*Glycine	-	-	>1.0×10^{-3}
*Glutamate	-	-	>1.0×10^{-3}

* non-cholinergic

remaining at various concentrations of displacing drug is expressed as a percentage of Bmax. The Hill coefficients calculated from the slope of the lines should be unity if the displacement is of a competitive type and at a single set of binding sites. Hill coefficients below unity (and shallow displacement curves) indicate multiple binding sites in the interaction of two ligands. This may well be true of the stereoisomers of nicotine and of carbachol, as judged by their effects on binding of [^{125}I] α-BGT to TE671 cells (Tables 7 and 8). d-Tubocurarine has been noted as having a shallow displacement curve in binding to TE671 cells (Syapin et al., 1982); displacement curves of a similar nature were not found in this study.

Methyllycaconitine (MLA), a naturally occuring toxin found in the seeds of Delphinium brownii, showed the lowest potency in inhibiting [^{125}I] α-BGT binding to TE671 cells. Further investigations showed it to have similarly low potency in inhibiting toxin binding to frog and human muscle preparations; Hill coefficients for these preparations were lower than for those found in TE671 cells (Fig. 22 and Table 9) probably as a consequence of proteolysis.

MLA is a potent insecticide (Jennings et al., 1986) and moderately effective as an antagonist of the AChR at the neuromuscular junction in the rat phrenic nerve diaphragm preparation (Nambi-Aiyar et al., 1979). MacAllan et al. (1988) found it to be a potent inhibitor of [^{125}I] α-BGT binding to rat brain (confirmed in this

study (Fig. 22 and Table 9)) and to locust ganglia but a poor inhibitor of binding to Torpedo AChR. These data suggest that MLA displays a higher affinity for the α-BGT binding site found in neuronal tissue than that found at the periphery (Table 17) and may be useful as a prefential probe for the neuronal toxin receptor of vertebrates and invertebrates. These results further confirm the similarities between TE671 AChR and that of muscle.

The inhibition of [^{125}I] α-BGT binding sites on TE671 cells by myasthenic serum was measured by determining the percentage of sites remaining after pre-incubation of the cells with myasthenic serum. Reductions, relative to normal serum controls were shown in the three patients tested. These effects were independent of complement as the sera were heat inactivated before use. Inhibition of [^{125}I] α-BGT binding was dependent on serum concentration for all the samples tested. As these assays were conducted at room temperature and 37°C, the results probably arose from increased energy-dependent receptor degradation, although direct blockade of the α-BGT binding site cannot be ruled out.

Theoretically, it is possible that antibodies could produce blockade of AChRs by binding at, or near to the active sites of the receptors, sterically hindering access to the sites, or by allosterically interfering with α-BGT binding (Figs. 44 and 45). It has been reported that only a small proportion of antibodies to

Table 17. Inhibition of [^{125}I] α-BGT binding to various tissues by MLA.

Tissue	Ki (M)	Reference
Rat brain	1.4×10^{-9}	MacAllan *et al.* (1988)
Torpedo	1.15×10^{-6}	"
TE671 cells	5.0×10^{-5}	This study
Human muscle	1.4×10^{-5}	"
Frog muscle	1.2×10^{-6}	"
Rat brain	1.1×10^{-9}	"

Fig. 44. Possible sites of attachment of anti-(AChR) antibody causing blockade of receptor.

a) Antibody attaches to acetylcholine binding site.

b) Antibody attaches near acetylcholine binding site and causes steric hindrance of acetylcholine and α-BGT binding.

c) Antibody does not directly interfere with the acetylcholine binding site, but alters the AChR molecule thus inhibiting acetylcholine and α-BGT binding.

FIG. 44 Possible sites of attachment of anti AChR antibody causing blockade of receptor.

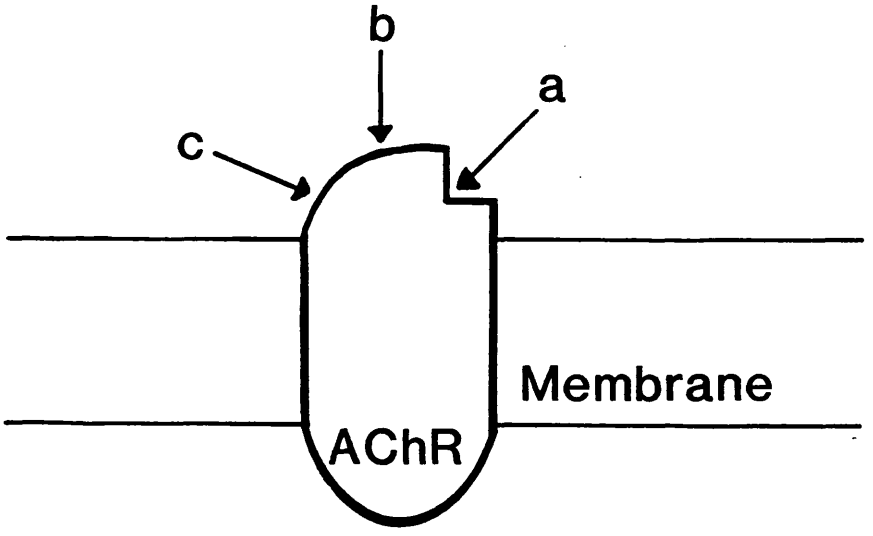


Fig. 45. Relative sizes of IgG, α-BGT, d-TC and acetylcholine with respect to the AChR.

Far right, AChR is depicted spanning the lipid bilayer.

IgG - white

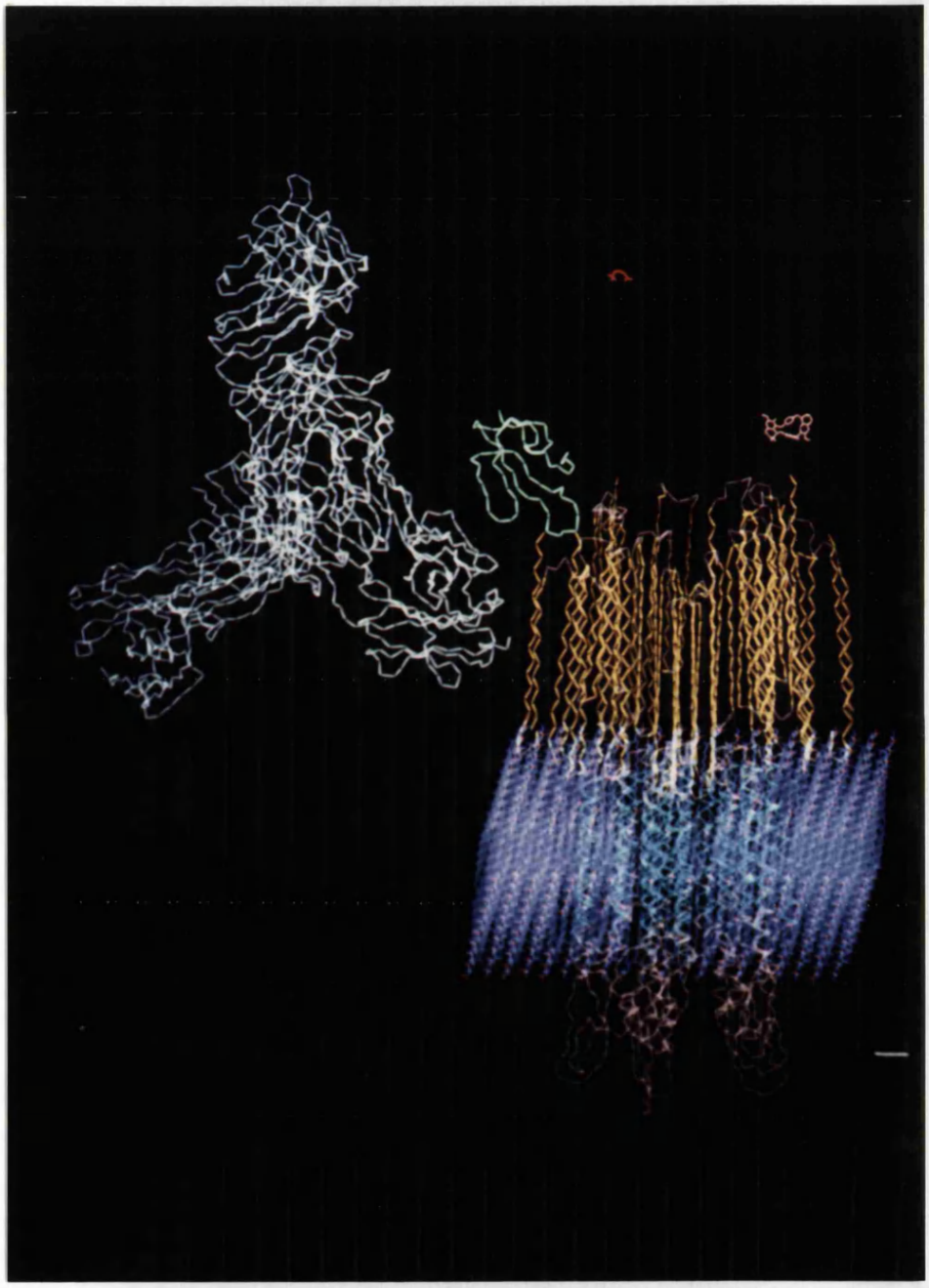
α-BGT - green

d-TC - pink

ACh - red

Molecular models constructed by V.B. Cockroft.

FIG. 45 Relative sizes of IgG, α-BGT, d-TC and acetylcholine with respect to the AChR.



the AChR in myasthenic patients bind directly to the α-BGT binding site itself (Dwyer et al., 1979; Mittag et al., 1981; Vincent and Newsom-Davis, 1979,1980,1982; Whiting et al., 1983), although in other studies using chick muscle cells in culture (Fulpius., 1981) nearly half of the patients had some antibodies capable of binding to the site. However, the ability of antibodies to block binding of α-BGT may not necessarily parallel their ability to interfere with acetylcholine binding at the neuromuscular junction.

4.4. TE671 cells for the investigation of complement-mediated lysis.

In view of the disadvantages (sporadic supply and ease of contamination) of human muscle cell cultures, TE671 cells were chosen as carriers of human muscle-type receptor (see Section 4.2.) for further investigations of complement-mediated cytotoxicity.

In the majority of cytotoxicity assays the target is prelabelled with radioisotope which is released upon lysis of the cell. However, enzyme release can provide a cheap and convenient alternative assay. Szekeres et al. (1981), reported a method of determining cytotoxicity by measuring the alkaline phosphatase activity released from human embryonic fibroblast cells and Korzeniewski and Callewaert (1983) found that LDH is released in sufficient amounts in short term cytotoxicity assays, providing a sensitive and quantitative measure of

natural cytotoxicity. As multiwell scanning spectrophotometers can measure large numbers of samples with a high degree of precision, the possibility of using a colour reaction as a monitor of complement-mediated cytotoxicity towards TE671 cells was investigated. The assay was based on those previously described (Bergmeyer and Bernt, 1974; Mossman, 1983; Korzeniewski and Callewaert, 1983; Decker and Lohmann-Mathes, 1988), but employed the tetrazolium salt, p-iodonitrotetrazolium violet, as the substrate. Activity of LDH in the cellular supernatant, monitored by optical density at 492nm, indicates the proportion of lysed cells.

Initial experiments tested the effects of normal and myasthenic serum samples, all of which had been heat-inactivated. All of the samples apparently caused release of LDH. The cytotoxicity was not enhanced by the addition of complement (Table 11 and Fig. 24) and correlation of anti-AChR antibody titre with cytotoxicity values was poor. In order to investigate this further, the cytotoxic effect of increasing concentrations of serum and GPC on the TE671 cells was studied. Variation was apparent between experiments (Fig. 25a-d) but there was still no difference between normal and myasthenic sera in the presence or absence of complement (Fig. 25e and f). The use of fresh myasthenic serum in one experiment showed marked cytotoxicity towards the cells but this was not repeatable in further experiments (Fig. 26) although it was observed that

cytotoxicity, if noticeable, was present at lower added concentrations of serum. The serum concentration range was further reduced and variation in the lower added range was again marked. This effect was attributed to the difficulties encountered in adding and adequately mixing very small quantities of serum in the 96-well culture plates without perturbing the monolayer.

The effect of heat inactivation versus non-heat inactivation of fresh serum samples on complement-mediated lysis also revealed no significant difference in cytotoxicity (Fig. 27a-d).

Investigations of the presence of endogenous LDH in normal and myasthenic sera did show background levels but these were considered to be low enough (20-50% of total LDH releasable from Triton lysed TE671 cells) so that increases due to complement mediated lysis could be measured.

LDH exists as a number of isoenzymes; LDH1 predominates in serum and LDH5 predominates in skeletal muscle (Table 18). In MG, cellular damage at the muscle end-plate may result in LDH5 release from skeletal muscle. If either or both of these isoenzymes in the serum samples could be inactivated specifically, the released LDH from TE671 cells could be more accurately measured.

LDH5 shows maximum activity at 42°C (Bergmeyer and Bernt, 1974). Temperatures above this result in denaturation. As the majority of serum samples were routinely heat-inactivated (56°C, 1h) before use or

Table 18. Three main types of LDH isoenzymes in tissues with representative examples.

Type	Predominant fraction	Example
Heart muscle	**LDH1**	heart muscle
		red blood cells
		kidney glomerular cells
		blood platelets
		eye lens
Skeletal muscle	**LDH5**	skeletal muscle
		liver
		kidney tubule cells
		granulocytes
		malignant tumours
Smooth muscle	**LDH3**	smooth muscle
		continuously contracting skeletal muscle

Taken from Bergmeyer and Bernt (1974)

storage, the LDH activity still detectable in serum samples was thought to be attributable to LDH1. Further heat inactivation of these samples (60-75°C, 1h) caused substantial, visible aggregation, rendering the samples useless for further assay.

The adsorption of serum samples onto two matrices, DEAE-Sephadex A-50 and Matrex Blue-gel A (Section 2.11.4.3.) was investigated as a means of specific removal of LDH1 (Bergmeyer and Bernt, 1974). In both cases serum LDH levels were reduced by about 50-65% (Figs. 30 and 31). However, both methods resulted in the concommittant reduction in anti-AChR antibody titre [10-20% loss with Sephadex A-50 and 30% loss with Blue-gel A (Table 12)]. Subsequent assays of lysis with Sephadex A-50 adsorbed serum samples, failed to demonstrate differences in lysis with myasthenic serum and GPC (Fig. 32).

From these results it was concluded that whilst levels of LDH in the serum could be reduced, the assay itself was not sensitive enough to monitor complement-mediated lysis of myasthenic serum towards the AChR on TE671 cells.

Further investigations of the cytotoxic effect of myasthenic serum on TE671 cells were carried out by using radiolabel. TE671 cells readily take up both L-[ME-^{3}H] carnitine and [^{51}Cr] (Figs. 33 and 34). However, TE671 cells are not contaminated by additional cell types in culture. The reason for choosing L-[Me-^{3}H] carnitine in the myotoxicity studies is to

differentiate lysis specifically from muscle cells, from that of any other cell types and so the cheaper [^{51}Cr] label was chosen in these studies.

The quantitation of immune cytolysis based on the detection of released [^{51}Cr] from lysed nucleated cells was introduced by Goodman (1961). This method has been useful and [^{51}Cr] fulfills the expected criteria of a marker for labelling a single population of target cells:- It is non-toxic, the release of radiolabel from the target is even, the detection is relatively easy and sensitive and, what is most important, it does not change the morphology and characteristics of the target cells (Blomberg et al. (1986).

Uptake of [^{51}Cr] by TE671 cells was not saturable over the range studied (0-2µCi/well and 0-20µCi/well) but levels of radioactivity taken up were adequate. Loss of radioactivity from the cultures was approximately 3%/hour, in agreement with the findings of Blomberg et al. (1986), who found also found that spontaneous release of [^{51}Cr] from cells is relatively low.

Preliminary experiments tested the effects of increasing concentrations of heat-inactivated myasthenic and normal serum samples on [^{51}Cr]-labelled TE671 cells. Cytotoxicity increased with increasing serum concentration, a decrease in cytotoxicity at high concentrations of normal human serum (80-90%, Fig. 37) was due to loss of cells from the culture plate. The addition of complement in further assays revealed variation between samples. However, cytotoxicity was

most marked at 20% serum.

Subsequent assays for cytotoxicity, employed 20% serum. For only one patient (MG2), was the combination of myasthenic serum and GPC found to be most effective at promoting lysis.

The specificity of the antibody-dependent complement-mediated cytotoxicity to TE671 cells was investigated by using an antiserum raised in rabbit against purified fetal calf AChR.

Using the same assay conditions as in the cytotoxicity of serum and GPC, rabbit anti-(fetal calf AChR) antiserum produced loss of [^{51}Cr] from TE671 cells, but only at the level of 15% lysis.

These results, with the experimental antisera suggest that "real" lysis with the serum was not being achieved, as levels for cytotoxicity were within the range defined as "control" by Childs et al. (1984, 1985, 1987). Whilst it was shown that TE671 cells could be lysed with water and the non-ionic detergent Triton X-100, the data presented suggest that TE671 cells are resistant to complement effects mediated by antibodies towards the AChR from either human or animal sources. Possible reasons for this lack of lysis could be:-

1)Low cell surface density of AChR on TE671 cells.

2)Ineffective source of complement.

3)Difficulty in achieving lysis by a complement-mediated mechanism of any nature.

It is known that TE671 cells have cell surface AChRs as demonstrated by high affinity [^{125}I] α-BGT

binding. However, the density of AChRs on TE671 cells is reported to be low (Sine, 1988). If this is true, complement activation may be inhibited in the manner described for fetal muscle cells in culture (Section 4.2.). Whether continuous repassaging of TE671 cells affects AChR properties or a reduction in actual numbers of binding sites is undocumented.

Ineffectiveness of the complement source was thought to be an unlikely reason, as the same complement, when coupled with myasthenic serum gave demonstrable lysis of human muscle cells in culture (Section 4.2.).

An investigation of other specific antibodies effecting complement-mediated lysis was not included in this study. This could, however, be studied using an alternative cell surface marker or protein as the antigenic target, or by preparing anti "TE671 cell membrane" or anti "TE671 cell" antibodies, for use with a known complement source.

In the myotoxicity and cytotoxicity experiments carried out for this project it is probable that the antibodies responsible for the observed lysis were directed at the AChR on the human myotube cultures and TE671 cell membranes. The effects of a specific anti-AChR antibody could be investigated using monoclonal antibodies raised against the native receptor. However, in addition to antibodies directed at the AChR, patients with MG have circulating antibodies to various other skeletal muscle antigens, although these antibodies

might not be directly involved in a lytic response, they may represent a large proportion of the total serum antibody population and their presence should not be ignored.

4.5. Involvement of other antibodies in MG.

Anti-striational antibodies directed against cytoplasmic elements of the contractile apparatus have been documented in MG (Newsom-Davis and Vincent, 1982). The presence of these antibodies was demonstrated before the involvement of anti-AChR antibodies was suggested (Strauss et al., 1960; Van der Gold et al., 1963; Beutner et al., 1966). Anti-striational antibodies are mainly associated with thymoma (Oosterhuis et al., 1976) but this is not specific for MG as 25% of patients with thymoma not associated with MG also have circulating anti-striational antibodies (Oosterhuis et al., 1976; Limburg et al., 1983). Further evidence for the involvement of non-"receptor" proteins as antigens in MG has been put forward. Evoli et al. (1989) found anti-filamin antibodies in patients with MG, however, these tended to be associated with occular MG rather than the generalised disease.

Non-receptor muscle antibodies in MG have often been regarded as specific for components of contractile proteins. Aarli et al. (1989) recently demonstrated binding of MG serum antibodies to the terminal cisterns of sarcoplasmic reticulum of striated muscle I bands.

They further investigated the antigenicity of fractions of rat muscle homogenate, finding that only fractions containing membrane proteins and not contractile proteins, showed a strong reaction with MG sera known to contain non-receptor antibodies (Aarli et al., 1989). These data suggest that the antibody repertoire in MG is very extensive and that all parts of the muscle may serve as autoantigens although it is unknown as to whether they are all pathogenic.

Myasthenic sera may contain antibodies against other cell surface determinants which could affect receptor activity indirectly. Evidence for the presence of such antibodies comes from a study by Muller and Anderson (1984) demonstrating the presence of antibodies against cell surface antigens on human medulloblastoma cells in the sera of patients with MG. That myasthenic sera contains antibodies against cells of neuronal origin suggests a more generalised neuroimmunological abnormality may exist in at least some patients.

In MG there is clearly a heterogeneous population of antibodies, not all of which are pathogenic. Disease state may be more closely related to sub-populations of anti-receptor antibodies or against particular determinants on the AChR.

4.6. Autoimmune response in MG.

The initiation of autoantibody production in MG remains terra incognita, although it undoubtedly does

not involve immunisation with a dose of exogenous AChR in adjuvant. There is some evidence that autoimmune activity against AChR takes place within the thymus gland. Willcox et al. (1984) noted an increase in B-cells capable of producing IgG, some of which were directed at AChR. They also noticed (Willcox et al., 1984) that thymic lymphocytes from myasthenic patients cultured in vitro produced anti-AChR antibodies. These findings suggest that the thymus glands from at least some myasthenic patients contain the elements required for an immune response. Although the thymus is not a major producer of the patient's anti-AChR antibody, it may well be an important site for the origin and maintenance of the autoimmune esponse in MG.

The association of thymic abnormalities with MG and the presence of antibodies directed against other muscle components suggest that the initiating mechanism could involve more than the AChR per se. Lentz et al. (1986) noticed that the rabies virus is capable of using the AChR as a tissue specific recognition site, Steffanson et al. (1985) showed that various common pathogenic bacteria can cross react with monoclonal antibodies directed against the AChR. These data imply that an immune process triggered by a viral infection or bacterial infection may induce an autoimmune response against the AChR.

Irrespective of how the autoimmune response is initiated, the question of how the response is maintained has yet to be answered. Under normal

circumstances, once initiated, an immune response tends to subside in the absence of antigen. Experimental administration of exogenous AChR initiates EAMG in animals (Patrick and Lindstrom, 1973; Heilbronn and Mattson, 1974) but maintenance of the autoimmune response requires repeated administration of AChRs (Drachman, 1987).

Various mechanisms have been proposed for immunoregulation and its breakdown. No-one can give a satisfactory explanation and the process is more complex than at first sight. However, an alteration in immunoregulation seems likely for the autoimmune response in MG (Fig. 46).

The possibility that there may be a deficiency of suppressor cells specific for anti-AChR antibody has been proposed (Shapiro et al, 1981; Richards et al., 1986). Suppressor T-cells are thought to down-regulate the immune response. Asherson et al. (1986) suggested that once AChR-specific suppressor cells are activated specifically, they secrete factors that non-specifically suppress the immune response. Drachman (1987), suggested that the occurence of antibodies against several antigens besides the AChR in some patients indicates that they may lack suppressor mechanisms for more than one antigen.

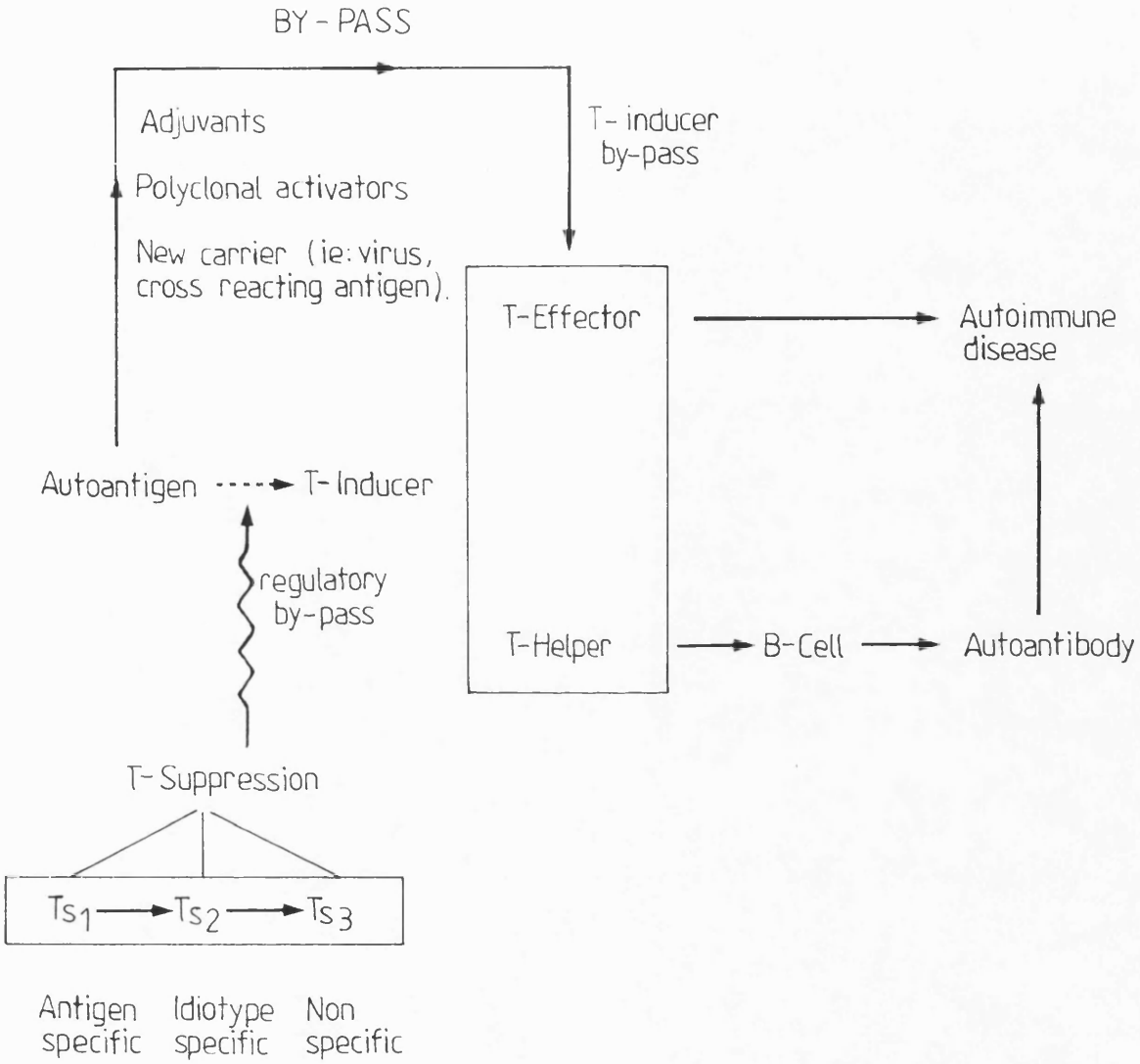
The production of autoantibodies is not limited to disease, but appears to be part of the normal B-cell repertoire. It is conceivable that the isotype of the antibody could be important in determining its capacity

Fig. 46. Some possible interactions in an immune response to self.

Autoimmunity arises through bypass of the control of autoreactivity. The constraints on the stimulation of self reactive inducer/helper T-cells by autoantigen can be circumvented either through by-passing the inducer cell or by disturbance of the regulatory mechanisms.

Taken from Roitt, 1987.

FIG. 46 Some possible interactions in an immune response to self.



to cause disease. Most natural autoantibodies are of the IgM class, while disease autoantibodies tend to be IgG (Cohen and Cooke, 1986). Thus an immunoglobulin class switch may be critical in the disease process (Cohen and Cooke, 1986).

The relative proportions of the IgG isotypes within the anti-AChR antibody population are important. Lefvert and co-workers (Lefvert and Bergstrom, 1987; Lefvert et al., 1981) and Rodgaard et al. (1987) noted a prevalence of IgG3 in anti-AChR antibodies, whereas other groups reported low levels of IgG3 (Vincent and Newsom-Davis, 1982; Whiting et al, 1983) with a predominance of IgG1 or IgG2 (Vincent and Bilku, 1982; Vincent and Newsom-Davis, 1982). The IgG isotypes vary in their ability to bind C1q and activate complement tightly (Burton et al., 1985), IgG1 and IgG3 being the most effective. However, storage of samples is important (Fulpius et al, 1981; Lefvert et al., 1981) as the immunoglobulins are susceptible to proteolytic degradation. The Fc region must remain intact to be functional in complement-related lytic investigations.

C3 is the central component of the complement pathway and measurement of its breakdown products reflects activation both the classical and alternate pathways. Most activated C3 reacts with water and is broken down to free C3d. C3d levels are thought to reflect the degree of complement activation in vivo (Charlesworth et al., 1974) and in vitro (Perrin et al., 1975) because C3d has the longest half life of the C3

breakdown products (Charlesworth et al., 1974). Monitoring C3d for elevation of disease activity and complement activation dynamics has considerable advantages. In particular, determination of C3d has been considered to be a valuable parameter in evaluating disease activity in patients with the autoimmune disease, rheumatoid arthritis especially those with extra-joint manifestations (Nydegger et al., 1977; Bedwell et al., 1986).

In a preliminary study to investigate an effect of C3d in MG and perhaps to correlate this with values obtained in the myotoxicity or cytotoxicity studies or with clinical state, use was made of a double-decker rocket immunoelectrophoresis technique developed by Brandslund et al. (1981) for investigating C3d activity in rheumatoid arthritis. With respect to reference preparations where C3 degradation was either prevented or spontaneous conversion of C3 in serum was allowed (37°C, 5 days), all of the myasthenic sera tested were positive and results did not correlate with titre (Fig. 41). It has been documented that activation and degradation of complement factors are influenced in vitro by temperature, time and the coagulation process (Sinosich et al., 1982). The EDTA samples used in this study had been stored, frozen (-80°C) for several months. This effect was noticed after the same samples (Fig. 41d) were re-assayed at Southmead Hospital, Bristol, U.K. and levels of C3d were found to be higher than for the previous assay (Fig. 41c). That

all of the serum samples tested were positive was considered unusual (R. Czudek, personal communication) but could be ascribed to storage or collection of the samples used.

It is likely that both complement-mediated attack and AChR modulation contribute to the AChR deficiency at the MG end plate. Lysis of the post synaptic membrane not only causes immediate loss of AChR, but also reduces the membrane surface available for insertion of new AChR. This in turn enhances the subsequent depletion of AChR by both modulation and complement. Modulation on the other hand, by decreasing the density of AChR and hence the antibody bound to AChR, may decrease complement fixation and thus help the post synaptic membrane escape destruction from subsequent membrane attack.

Conclusions and future prospects.

Previous investigations in this laboratory have successfully demonstrated complement-mediated lysis of myasthenic serum towards rat muscle cells in culture (Childs et al., 1984,1985,1987). This study investigated the same effect on human tissue and cell culture systems, proposing that they would serve as a better model for a human disease.

Human fetal muscle cells in culture showed myotoxicity but only at high levels of added serum and complement. Such an effect may be related to immaturity

of the cells in culture. Lysis of TE671 cells was demonstrated using either distilled water or Triton X-100 but a specific lytic effect of serum and complement was not apparent. These data suggest that TE671 cells have very resistant cell membranes.

The nature of the antibodies producing lytic activity and the role of complement require further investigation.

A more detailed examination of the IgG isotypes within patients, coupled with complement activation monitored via measurement of C3d could provide greater insight into the involvement of complement in MG.

TE671 cells are a good source of AChR but results from this study suggest that they are not a suitable cell type for the investigation of complement-mediated lysis.

It has very recently been suggested (Larsson and Sjoquist, 1989) that an assay measuring cell lysis is probably not the most appropriate method for use in patients with suspected autoimmune diseases, since it is mainly deficiencies of the classical complement pathway components that are associated with these diseases. This, however, does not dismiss the use of TE671 cells to benefit further study of MG and aspects of the AChR.

MG is the best characterised autoimmune disease of the nervous system. Several important aspects of MG are clearly known. It is clear that neuromuscular transmission is impaired as a result of several antibody mediated mechanisms in response to muscle AChRs. Assay

of the antibodies produced can improve diagnosis. Advances in the study of AChRs and immunology have permitted greater definition of the antigenic structure of the AChR and the cellular interactions involved in the production of antibodies.

If the pace of new development, with respect to molecular biology and immunology continues, a specific cure rather than palliative treatments may provide MG patients with a more acceptable quality of life.

APPENDIX

Table 19. Sera used in the myotoxicity study.

Serum No.	Result section number	% Myotoxicity (This study)	% Myotoxicity (Childs,1985)
1	3.1.4.	49	55
2	"	30	47
3	"	32	60
4	"	33	nd
5	"	35	nd

Table 20. Sera used in the cytotoxicity study.

Serum No.	Result section number		% Cytotoxicity (This study)	% Myotoxicity (Childs, 1985)
	LDH	[^{51}Cr]		
1	3.2.4.2.	3.2.6.4.	-	55
	3.2.4.3.	3.2.6.5.	-	
	3.2.4.4.	3.2.6.7.	-	
	3.2.5.1.		-	
2	3.2.4.3.	3.2.6.7.	-	47
3	3.2.4.4.		-	60
	3.2.4.6.		-	
	3.2.5.1.		-	
	3.2.5.2.		-	
	3.2.5.6.		-	
6	3.2.4.3.	3.2.6.7.	-	nd
	3.2.5.1.		-	
7	3.2.4.3.		-	nd
8	3.2.4.4.		-	nd
9	3.2.4.4.		-	nd
	3.2.4.6.		-	
10	3.2.4.5.		-	nd
11	3.2.4.5.		-	nd
	3.2.5.1.		-	nd
12	3.2.4.7.	3.2.6.7.	-	nd
	3.2.5.1.		-	
	3.2.5.2.		-	
	3.2.5.6.		-	
	3.2.5.7.		-	
13	3.2.4.7.		-	nd
	3.2.5.1.		-	
	3.2.5.2.		-	
	3.2.5.6.		-	
14		3.2.6.7.	-	nd

Table 21. Sera (EDTA-plasma) used in C3d assays.

Serum No.	Result section number
2	3.3.1.
3	"
10	"
15	"
16	"
17	"
18	"

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